THE AMERICAN JOURNAL OF PATHOLOGY

VOLUME XXX MARCH-APRIL, 1954 NUMBER 2

THE PERMEABILITY OF LUNG PARENCHYMA TO PARTICULATE MATTER *

PAUL GROSS, M.D., and MARIAN WESTRICK, PH.D.

(From the Industrial Hygiene Foundation, Mellon Institute, Pittsburgh 13, Pa.)

The manner in which dust particles are transported from alveoli into the pulmonary parenchyma and lymph nodes has been the subject of controversy for over 80 years. During the past 50 years opinions in regard to this mechanism have become largely set, with only a few dissenting. The prevailing concept is that macrophages emigrating to the free surface of the respiratory membrane engulf dust particles and then immigrate into the parenchyma. This opinion is recorded by most authors of standard textbooks.[1-10] The contrary opinion, that dust particles *per se* penetrate the pulmonary parenchyma and are carried by tissue fluid and lymph to their destination, finds support in but a few texts.[11-13] Several authors[14-16] adopt a "middle of the road" opinion and state that both mechanisms are operative.

Some of the earlier writers who argued for direct penetration of dust particles into lung tissue were Slavjansky[17] (1869), Ruppert[18] (1878), and Arnold[19] (1885). In more recent times Drinker[20] (1922) maintained that certain silicates may penetrate directly into the pulmonary parenchyma. Cameron and Lang[21] (1933) denied that emigrating macrophages immigrated back into the lung tissue, while Robson, Irwin, and King[22] (1934) held that silica particles penetrated the lung substance without the aid of phagocytic cells.

The theory of the "emigrating-immigrating" macrophage was supported by v. Ins[23] (1878), Schottelius[24] (1878), and Shingu[25] (1910). In 1914 Klotz[26] stated, "It would appear, however, with the more recent studies that the migration of the dust particles from the air sacs occurs only through the agency of certain wandering cells." Per-

* Presented at the Fiftieth Annual Meeting of the American Association of Pathologists and Bacteriologists, St. Louis, April 2, 1953.

Received for publication, July 25, 1953.

mar[27] in 1920, gave this concept the much needed and widely quoted experimental support. Other supporters of this view include Haynes[28] (1931), Gardner[29] (1932), and Belt[30] (1934).

A number of writers espoused both concepts. They are Knauff[31] (1867), Slavjansky[17] (1869), Ruppert[18] (1878), Lemon and Higgins[32] (1932), Robertson[33] (1941), and Drinker[34] (1952).

During microscopic studies of the lungs of animals subjected to experimental pneumoconioses by inhalation as well as by intratracheal injection, large numbers of extracellular particles were noted in interstitial positions. The existence of the extracellular particles in such sites was difficult to explain on the basis of the prevailing concept. Furthermore, it was recalled that in many sections of lungs from human necropsy material the arrangement of interstitial anthracotic pigment deposits suggested that the material was extracellular. It was noted frequently that assumptions of an intracellular position of the pigment were arbitrary and rested on very tenuous grounds.

The theory that particles *per se* may penetrate the lung substance seemed intriguing and appeared to have support in past experimental observations. The following experiments were designed to test this possibility.

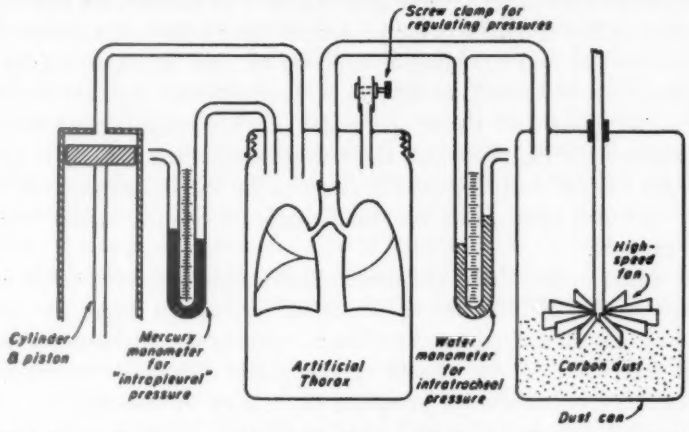
Materials and Method

In Vitro Experiments

Rats were killed with ether. The lungs with trachea attached were removed and suspended within a small glass jar provided with a screw-on metal cap (Text-fig. 1). In the cap four small metal tubes were soldered. The trachea with lungs attached was tied to the central cannula. A second tube was connected to a cylinder with piston (displacement, 25 cc.) actuated by an electric phonograph motor at the rate of 78 cycles per minute. A manometer for measuring the "intrapleural" pressure was connected with the third tube; the fourth cannula was provided with a short piece of rubber tubing, the opening of which was controlled with a screw clamp for regulating the "intrapleural" pressure by manipulating the amount of leakage into and out of the glass jar. The tracheal cannula was connected by a T-tube to a large can containing carbon dust * (0.14 μ, average particle size). The air in this can was heavily charged with the carbon dust by means of turbulence created by a rapidly revolving stirrer of fan type. The other end of the T-tube was connected to a water manometer to measure the intratracheal pressure. Movement of the piston within the cylinder caused the lung alternatingly to "inhale" and "exhale"

* P-33, obtained from the Columbian Carbon Co., Brooklyn, New York.

carbon-dust-charged air from the can with the simultaneous registration of "intrapleural" and intratracheal pressures. Such "respirations" were continued from several minutes to several hours in different sets of lungs under different pressures. At the end of each experiment the lungs were inflated with formalin under a pressure of 12 cm. of water and prepared for sectioning. All paraffin sections from this and



Text-fig. 1. Diagram of apparatus for the mechanical respiration of an excised lung and for supercharging the respired air with dust.

subsequent experiments were exposed to ammoniacal alcohol for a minimum of 8 hours to avoid artifactual pigment deposits caused by formalin.

Intratracheal Injection of India Ink

India ink was diluted with equal parts of saline solution and injected intratracheally into rats in 1 ml. doses. Although electron photomicrographs were not made to determine particle size, it appeared from a study of a very dilute suspension of India ink that the particle size was near or below the limit of resolution of the optical microscope, that is, 0.2 μ. The animals were killed with ether 4 hours to 4 days later and the lungs were inflated with formalin.

Results

In Vitro Experiments

It was noted that while "respiratory" excursions were obtained with relatively low pressure gradients for the first few cycles at the beginning of the experiment, these excursions rapidly became smaller with subsequent cycles while the intratracheal pressure simultaneously diminished. In order to maintain adequate excursionary activity of

the lungs it was necessary to increase the "intrapleural" pressures to ± 4 cm. of Hg. However, the intratracheal pressure remained fairly constant at ± 8 cm. of water.

The most successful experiment lasted 4 hours at "intrapleural" pressures of ± 4 cm. of Hg. At the end of this time the lungs were completely black externally and on section. No pink tissue was discernible. Sections of such lungs showed large amounts of coarse flocs and finely divided carbon granules in the air spaces. In addition, the lining from bronchioles to and including alveoli was covered in many regions by a closely applied, thick membrane of homogeneous, agglutinated carbon. All interstitial tissues presented an extremely severe edema, throughout the fluid of which there were scattered innumerable finely divided carbon particles, giving portions of the edematous tissue a gray to black tone. This was particularly pronounced about vessels and bronchi.

It would appear from the gross observations and microscopic sections that shortly following the induction of artificial respiration there occurred in the excised lungs a shift of fluids to the interstitial tissues. This interstitial edema caused turgidity and increased the pressure differentials necessary for adequate respiratory movements.

Intratracheal Injection of India Ink

After intratracheal injection the distribution of India ink grossly was diffuse and fairly uniform. Occasionally an upper lobe showed a gray discoloration in contrast to the black elsewhere. In rats sacrificed 4 hours following the intratracheal injection, there was considerable edema of perivascular and peribronchial stroma with scanty infiltration of polymorphonuclear leukocytes. There was neither edema nor exudate in the alveolar spaces, although the alveolar capillaries were dilated and congested. The pigment was disposed in a thick layer covering the walls of the air spaces, in places becoming thinner. Frequently it was interrupted by fissures. In many regions the pigment masses were separated from the wall of the air space, leaving only fine granules adherent to it. Very few isolated alveolar macrophages were present. Pigment granules were present within or upon the surfaces of the cells. Much of the pigment appeared "fractured"; and scattered, coarse pigment granules as well as larger aggregates appeared smeared or dragged over the tissues without regard to anatomical structures. There was no evidence of true penetration of pigment into the pulmonary stroma.

The lungs, 19 hours following the injection, were grossly identical to those previously described. Microscopically, however, notable dif-

ferences were present. There was now well defined alveolar edema as well as moderate exudation by polymorphonuclear leukocytes. The alveolar lining cells were increased in number and slightly swollen. Perivascular and peribronchial edema was pronounced and more leukocytes had infiltrated the stroma. The injected pigment appeared as a closely applied, dense membrane covering the walls of the air sacs. The fissuring and fracture-like fragmentation of the pigment seen earlier were not present. Some of the pigment was seen in the center of the alveoli and ducts, apparently mixed with edema fluid, forming a web-like stringy matrix, in the interstices of which many leukocytes and a few macrophages were present. Some of these cells were covered with, or contained, pigment granules. The pigment material throughout was well delimited by anatomical structures and there was no evidence of artifactual scattering of pigment as was seen in the rats killed earlier. The pigment material gave the impression of being a cohesive, as well as adhesive, fluid mass in contrast to the brittle, less adhesive character of the injected pigment seen 4 hours following the intratracheal injection.

Where alveoli were contiguous with larger vessels and bronchi there were widely scattered small foci showing penetration of pigment into the edematous stroma. Many of these foci were less than one fourth of the diameter of an alveolus in width and were recognizable under low magnification by a gray cast or darker tone of the tissues. Under high magnification many ultra-fine and a few larger pigment particles were found clinging to the edges and surfaces of connective tissue fibrils. These particles accentuated the fibrils, often giving them a beaded appearance. Some granules were clinging also to the surface of nearby cells. Discrete pigment granules and a few small aggregates were present between connective tissue fibrils, apparently suspended in edema fluid to form a slurry. Occasionally, the slurry was sufficiently dense to obscure the fibrils partially. An occasional polymorphonuclear leukocyte containing black pigment was present within or near such regions.

Perhaps the most convincing feature about these observations was the fact that such foci were well defined and could be identified in two or three successive serial sections as occupying exactly the same relative position. Some of the foci exhibited a fan-shaped spread of fine particles with the apex of the fan near the respiratory membrane. An obvious tear or other interruption in continuity of the membrane could not be demonstrated. Very few pigment particles were seen at any great distance from the respiratory membrane.

Sections of the lungs from a rat killed 4 days after intratracheal

injection of India ink showed almost complete replacement of the leukocytes by macrophages. Much of the alveolar pigment was now intracellular. Alveoli were lined by swollen, epithelium-like cells. The perivascular and peribronchial stroma was still edematous but now contained numerous cells with ovoid nuclei and ill defined cytoplasmic processes. A few eosinophils and scattered neutrophils were present also. There was much pigment scattered about in the stroma but no longer in finely divided form. The pigment was agglomerated to form masses which were frequently too large and too irregular to constitute pigment-laden cells. Although occasional pigment clumps were found related to cells lying either on the surface or within the cytoplasm (a point difficult to establish), there were also numerous small and larger aggregates which were obviously extracellular and lying free or adherent to fibrils. A few such aggregates had actually reached the proximity of the adventitia of vessels. Most of the cells in the stroma resembled the fixed tissue cells rather than wandering cells.

The extracellular alveolar pigment still retained its stringy and web-like character but the interstices were larger and more numerous. The impression was gained from these sections that the cohesive, viscid character of the pigment material was maintained. Indications of artifactual fragmentation or smearing of the pigment were absent.

Rats which died of pneumonia 18 to 48 hours following intratracheal injection showed no penetration of particulate matter.

No interstitial pigment was found in uninjected control animals.

Discussion

The massive invasion of the pulmonary parenchyma by carbon dust in the excised lung under the conditions of these experiments is, no doubt, influenced by post-mortem changes. There is little question that the permeability of membranes undergoes radical changes immediately after death and with the lapse of time autolytic changes contribute to the destruction of the anatomical integrity of membranes. It is probable that the duration of the most successful of the lung preparations, 4 hours, was sufficient to allow for a considerable degree of such alteration. However, the migration of particulate matter into the perivascular and peribronchial tissues and its tendency to concentrate about these structures cannot be considered artifactual and must be attributed to the fluctuations in intrapulmonary pressures and to the excursionary activity of the lung tissue.

It may be argued that the excessive pressures ruptured the alveolar lining and that the carbon particles were carried into the interstitial tissues by inrushing air which produced an interstitial emphysema.

In rebuttal it should be pointed out that the interstitial emphysema is as distinctive in the lung as it is in other tissues. This was ascertained by introducing a single, short blast of air under very high (not measured) pressure into the trachea of three freshly excised rat lungs. The lungs were then inflated with formalin and sections prepared. The air bubbles in the interstitial tissues, with their sharply circumscribed, circular periphery and empty content, presented an entirely different appearance from the edematous spaces seen in the lungs from *in vitro* experiments. The latter were irregular in configuration and contained fibrillar fragments or pink-staining homogeneous material, or both.

The migration of particles in the excised lungs suggests that once penetration of the alveolar membrane is accomplished in the living lung the particles may be propelled to perivascular and peribronchial regions without the aid of cells. The particles are, no doubt, assisted in this migration by the flow of tissue fluid if this flow is in the same direction, which is the case under normal conditions and during the subsiding or resorptive phase of an inflammation. During the exudative phase of an inflammation the flow of tissue fluid is toward the alveoli and the migration of particles is retarded or entirely inhibited, depending upon the force of the edema fluid.

The presence of discrete extracellular carbon particles in the perivascular and peribronchial stroma of rats which received intratracheal injections of India ink 19 hours to 4 days prior to their sacrifice is considered proof that particulate matter may penetrate *per se* beyond the respiratory membrane without the mediation of phagocytic cells. Free carbon particles in the pulmonary stroma were not nearly as obvious as in the *in vitro* experiments. Not only was the penetration of extracellular particles quantitatively very much smaller, but it also lacked the diffuseness seen in the excised lungs. The more or less localized character of the penetration in the living animal, mainly about larger vessels and bronchi, suggests that this phenomenon is associated with focal defects in the respiratory membrane, such as erosions.

Whereas there can be no question regarding the authenticity of the interstitial carbon particles in sections from the *in vitro* experiments because of the diffuseness and massiveness of the material, some doubt may arise when particles are relatively few, widely separated, and limited to a few small foci. It is therefore important to rule out artifactual processes as factors in the production of such particles. Formalin precipitate can be safely ruled out because of the prolonged treatment of the sections with ammoniacal alcohol. The fortuitous

dragging of particles or "smearing" by the microtome knife is eliminated as a possibility by the demonstration of clusters of extracellular particles within the pulmonary stroma occupying exactly the same relative position in two or more successive serial sections.

Attempts were made also to study the penetration and migration of particles in rats breathing very heavy clouds of carbon dust for about 30 hours over a period of 4 days. The amount of dust which reached the bronchial passages within this time was amazingly small, attesting to the efficiency of the nasal passages as a dust filter. Only occasional flocs and rare particles of carbon were found in the air spaces. Search with an oil-immersion lens disclosed very few, widely scattered, isolated and extracellular carbon particles within alveolar walls and within the interstitial tissues about bronchi and vessels, but near the alveolar lining. Although such findings are far from convincing because of their paucity, the possibility exists that particles which are sufficiently small may penetrate the intact alveolar lining without interrupting its continuity. There is great need for information regarding the reciprocal behavior of ultrafine particles and serous membranes.

The same factors which contributed to the interstitial dispersion of pigment particles in the *in vitro* experiment are considered operative in the living animal injected intratracheally with India ink; namely, fluctuations in the respiratory pressure and the respiratory excursions of lung tissue.

The failure to find evidence of penetration of particulate matter into the pulmonary parenchyma in many animals dying quickly (within 24 to 48 hours) following an intratracheal injection of particulate matter is probably attributable to the forceful opposition of edema fluid. This fluid, up to the time of death, was streaming into the alveoli. Other important factors are the vitiation by the alveolar edema fluid of the pressure effects of respiration upon the lung stroma and the inhibition of respiratory excursions by the same fluid.

The reason for the apparent difference in the physical character of the injected pigment at 4 hours following injection, as compared to 19 hours, is a matter of conjecture. One plausible explanation is that immediately following the intratracheal injection there is resorption of the injected fluid resulting in inspissation of the pigment. Later, edema develops and by virtue of the respiratory excursions the pigment is thoroughly mixed with fluid of high protein content which gives the material its viscid, cohesive as well as adhesive character.

In conformity with the theory that macrophages are the sole means of transport of pigment into the lung substance, it is generally held that anthracotic deposits within the lung are intracellular with the

exception of pigment which may have been liberated by the disintegration of a macrophage. Without implying that pigment-laden macrophages may not occur in or near anthracotic deposits, a more critical attitude will disclose that decisions regarding the intracellular position of pigment are based largely upon conjecture and are not susceptible of demonstration. Cell outlines are not distinguishable and nuclei generally cannot be seen. Only occasionally and in regions where the deposits are less homogeneous or less dense an isolated nucleus may be discernible. The determination as to whether such a nucleus is that of a macrophage or of a lymphocyte or even of a fixed connective tissue cell is fraught with great difficulty and any judgment would seem to be arbitrary. It is not rare to find a sharp edge of a pigment mass subtending a portion of a nucleus like a chord, or lying tangent to it and continuing beyond in a straight line so as to suggest that the pigment mass overlies the cell. Certainly, in view of the frequently considerable size of the masses the existence of connective tissue cells and lymphocytes lying within, over, and under the masses need not be doubted.

While the periphery of deposits frequently exhibits a scalloped outline and often a small convexity at the periphery could be interpreted as representing a cell, more commonly such formations are too large and too irregular to represent cells. Scrutiny of anthracotic deposits in areas of pneumonia or edema in which disintegration[35] of the deposits is in progress generally discloses no phagocytic cells or recognizable remnants of macrophages within the confines of the deposits. It is true that occasionally macrophages can be observed peripheral to the areas of disintegration, but these cells contain discrete fine and coarse pigment as well as distinctly discernible cytoplasm and well-staining nuclei, thus indicating that they are probably cells which had only recently taken up pigment. It is extremely doubtful that such macrophages are transformations[36] of the hypothetic cells which are presumed to comprise the deposits.

Review of lung sections from experimental pneumoconioses produced in this laboratory with Fe_2O_3, Al_2O_3, Sb_2O_3, and carbon resulted in similar conclusions. The interstitial pigment was aggregated to form dense, opaque deposits, some of which resembled cells in size and form, but most of them were too large and too irregular for such assumption. In sections in which disintegration[35] of pneumoconiotic deposits had occurred and the aggregates were broken up and separated by edema fluid, macrophagic constituents could not be identified.

Rats which had received intratracheal injections of finely divided

pottery clay presented a remarkably different picture. Although many interstitial deposits of some magnitude were present, the granules were always finely divided and within large macrophages. These cells had clearly defined cell boundaries, very pale, finely reticulated cytoplasm, and small, round, central nuclei. Dense, opaque aggregates were not found. The reason for this difference is not apparent and requires investigation.

The theory of the "emigrating-immigrating" or "returning" macrophages owes its popularity largely to the experimental work of Permar[27] who modified Slavjansky's[17] experiments. Briefly, Permar found that following the intravenous injections of isamine blue into rabbits, a few of the pulmonary capillary lining cells contained blue dye particles. When, immediately following the conclusion of a series of daily intravenous injections with this dye, carmine was injected intratracheally, the pulmonic stroma of rabbits sacrificed at intervals was found to contain macrophages which carried both red and blue pigment granules. Phagocytic cells containing both types of pigment were noted apparently in the process of traversing the respiratory membrane. Permar emphasized the necessity of injecting relatively large amounts of carmine as an irritant to demonstrate this phenomenon. He was unable to demonstrate any extracellular particles in the pulmonary stroma at any time.

Permar[27] reasoned that cells containing the blue dye could have originated only from the lining of the pulmonary capillaries and the cells which contained the red dye could have engulfed the particles only while they were on the luminal side of the respiratory membrane. Therefore, cells found within the pulmonary stroma which contained both kinds of dye must have emigrated from the pulmonary capillaries into the alveolus and, after engulfing carmine particles, immigrated into the stroma.

This conclusion was apparently based upon the premise that the wall of the pulmonary capillary bed is impermeable to isamine blue under the conditions of the experiment and that the respiratory membrane is similarly impermeable to carmine particles.

It is highly probable that under normal conditions such impermeability exists. However, the inflammatory reaction produced by the intratracheal injection of an irritant (carmine) makes an assumption of impermeability unlikely since vessels at the site of inflammation lose their semipermeability and become permeable. Also, it might be expected that multiple defects (erosions?) develop in the respiratory membrane under such circumstances. Thus it would seem that better and more convincing supportive data than is available at present

should be required for the continued acceptance of the theory of the "emigrating-immigrating" or "returning" macrophage. Aside from the pitfalls in the experimental basis for this theory, there are a number of theoretic considerations which are unfavorable to its continued acceptance.

A cell on the free surface of the respiratory membrane is properly considered to be outside of the body. It is difficult to match the re-entry of an inflammatory cell into the lung stroma with similar behavior of an inflammatory cell on any other body surface. Such re-entry is a feat of major proportions since a cell within the alveolus is deprived to some extent of its nutritive medium; also, having ingested foreign material it is generally enlarged and frequently has suffered damage as a result of the phagocytosis. Furthermore, it must insinuate its enlarged body against the positive pressure of the tissues. One might also marvel at the strange type of tropism which is positive prior to the ingestion of foreign material and negative thereafter.

It may appear strange that penetration and migration of particulate matter into the lung stroma has not been reported previously. The large number and variety of experimental investigations dealing with the pneumoconioses which have been reported during the past 85 years lend emphasis to this singularity. There appear to be a number of reasons for this. The most commonly used animal has been the rabbit, with the guinea-pig and the dog in second and third place, respectively. Rabbits, in this laboratory, have been found to react with a much more violent pneumonia to an intratracheal injection which, in the rat, evokes only a relatively mild response. Perhaps another important factor is timing. If the lungs of animals are examined during the exudative phase of the inflammation resulting from an intratracheal injection of particulate matter, little or no penetration is demonstrable. If, on the other hand, too much time is allowed to elapse following the injection, migration will be largely completed and aggregation or phagocytosis will have occurred. Other factors important to a satisfactory demonstration are sufficient fineness of the particles (less than 0.5 μ), adequacy of the amount and distribution of particulate matter within the alveoli, and adequate visibility of the particles.

Summary

It has been possible to demonstrate that particulate matter *per se* may penetrate the respiratory membrane and migrate to perivascular and peribronchial positions without the mediation of phagocytic cells.

Penetration of the respiratory membrane occurs in multiple, small, scattered foci near the apposition to alveoli and larger vessels and

bronchi. Although not demonstrated, it is nevertheless probable that the penetration of the respiratory membrane by particulate matter is dependent upon defects in the membrane, possibly in the form of erosions. Direct penetration of the alveolar lining by sufficiently fine particles is also a possibility.

Important physical factors which make penetration of the respiratory membrane and the migration of particles possible are the fluctuating pressures of the respiratory cycle and the respiratory excursions of the lung tissue.

On the basis of present experimental observations it would appear that most interstitial pneumoconiotic pigment deposits are probably extracellular agglomerations of particles.

Assertions that pigment had been carried to its interstitial pulmonary position by macrophages are conjectural and are based upon a theory which is physiologically unsound. The experimental support of this theory is founded upon a premise which is unacceptable in the light of our present knowledge. More convincing supportive data should be required for the continued acceptance of the theory of the "emigrating-immigrating" or "returning" macrophage.

REFERENCES

1. Delafield, F., and Prudden, T. M. A Handbook of Pathological Anatomy. W. Wood & Co., New York, 1901, ed. 6, p. 703.
2. McFarland, J. A Text-Book of Pathology. W. B. Saunders Co., Philadelphia, 1904, p. 539.
3. Adami, J. G., and McCrae, J. A Text-Book of Pathology. Lea & Febiger, Philadelphia, 1914, ed. 2, pp. 292, 549.
4. Karsner, H. T. Human Pathology. J. B. Lippincott Co., Philadelphia, 1926, pp. 14–15.
5. Boyd, W. The Pathology of Internal Diseases. Lea & Febiger, Philadelphia, 1940, ed. 3, pp. 166, 168.
6. Forbus, W. D. Reaction to Injury. The Williams & Wilkins Co., Baltimore, 1943, p. 549.
7. Moore, R. A. A Textbook of Pathology. W. B. Saunders Co., Philadelphia, 1944, p. 644.
8. Gunn, F. D. The Lung. In: Anderson, W. A. D. Pathology. The C. V. Mosby Co., St. Louis, 1948, p. 721.
9. Cameron, G. R. Pathology of the Cell. Charles C Thomas, Springfield, Ill., 1952, p. 378.
10. Bell, E. T. A Text-Book of Pathology. Lea & Febiger, Philadelphia, 1952, ed. 7, pp. 141–142.
11. Rindfleisch, E. Lehrbuch der pathologischen Gewebelehre. W. Engelmann, Leipzig, 1886, ed. 6, p. 432.
12. Ziegler, E. Lehrbuch der allgemeinen Pathologie und der pathologischen Anatomie. G. Fischer, Jena, 1906, **2**, 774.
13. Kaufmann, E. Pathology. (Translated by S. P. Reimann.) P. Blakiston's Son & Co., Philadelphia, 1929, **1**, 418–419.

14. Stengel, A. A Text-Book of Pathology. W. B. Saunders Co., Philadelphia, 1898, pp. 83–84, 437–438.
15. MacCallum, W. G. A Textbook of Pathology. W. B. Saunders Co., Philadelphia, 1940, ed. 7, p. 138.
16. Smith, L. W., and Gault, E. S. Essentials of Pathology. The Blakiston Co., Philadelphia, 1948, ed. 3, p. 411.
17. Slavjansky, K. Experimentelle Beiträge zur Pneumokoniosis-Lehre. *Virchows Arch. f. path. Anat.*, 1869, **48**, 326–332.
18. Ruppert, H. Experimentelle Untersuchungen über Kohlenstaubinhalation. *Virchows Arch. f. path. Anat.*, 1878, **72**, 14–36.
19. Arnold, J. Untersuchungen über Staubinhalation und Staubmetastase. F. C. W. Vogel, Leipzig, 1885, 204 pp.
20. Drinker, C. K. Modern views upon the development of lung fibrosis. *J. Indust. Hyg.*, 1922, 3, 295–303.
21. Cameron, G. C., and Lang, J. Immediate and ultimate effects of silica in the rabbit. *Tr. Roy. Soc. Canada*, 1933, **27**, s. 3, 173–176.
22. Robson, W. D., Irwin, D. A., and King, E. J. Experimental silicosis: quartz, sericite and irritating gases. *Canad. M. A. J.*, 1934, **31**, 237–245.
23. v. Ins, A. Einige Bemerkungen über das Verhalten des inhalierten Staubes in den Lungen. *Virchows Arch. f. path. Anat.*, 1878, **73**, 151–153.
24. Schottelius, M. Experimentelle Untersuchungen über die Wirkung inhalierter Substanzen. *Virchows Arch. f. path. Anat.*, 1878, **73**, 524–550.
25. Shingu, S. Über die Staubinhalation bei Kindern. *Virchows Arch. f. path. Anat.*, 1910, **200**, 207–220.
26. Klotz, O. Pulmonary anthracosis—a community disease. *Am. J. Pub. Health*, 1914, **4**, 887–916.
27. Permar, H. H. An experimental study of the mononuclear phagocytes of the lung. *J. M. Research*, 1920–21, **42**, 9–32.
28. Haynes, F. Experimental dust inhalation in guinea-pigs. *J. Hyg.*, 1931, **31**, 96–123.
29. Gardner, L. U. Studies on experimental pneumokoniosis. VIII. Inhalation of quartz dust. *J. Indust. Hyg.*, 1932, **14**, 18–38.
30. Belt, T. H. The pathology of pneumonoconiosis: A review. *Am. J. M. Sc.*, 1934, 188, 418–437.
31. Knauff. Das Pigment der Respirationsorgane. *Virchows Arch. f. path. Anat.*, 1867, **39**, 442–475.
32. Lemon, W. S., and Higgins, G. M. Pulmonary fibrosis: experiments of short duration. *Am. J. M. Sc.*, 1932, **183**, 153–164.
33. Robertson, O. H. Phagocytosis of foreign material in the lung. *Physiol. Rev.*, 1941, **21**, 112–139.
34. Drinker, C. K. Personal communication.
35. Gross, P., and Brown, J. H. U. The mobility of pneumoconiotic deposits. *Am. J. Clin. Path.*, 1952, **22**, 821–832.
36. Haythorn, S. R. Experimental Edema as an Aid to Histopathologic Studies. In: Contributions to Medical Science, Dedicated to Aldred Scott Warthin. George Wahr, Ann Arbor, 1927, pp. 491–502.

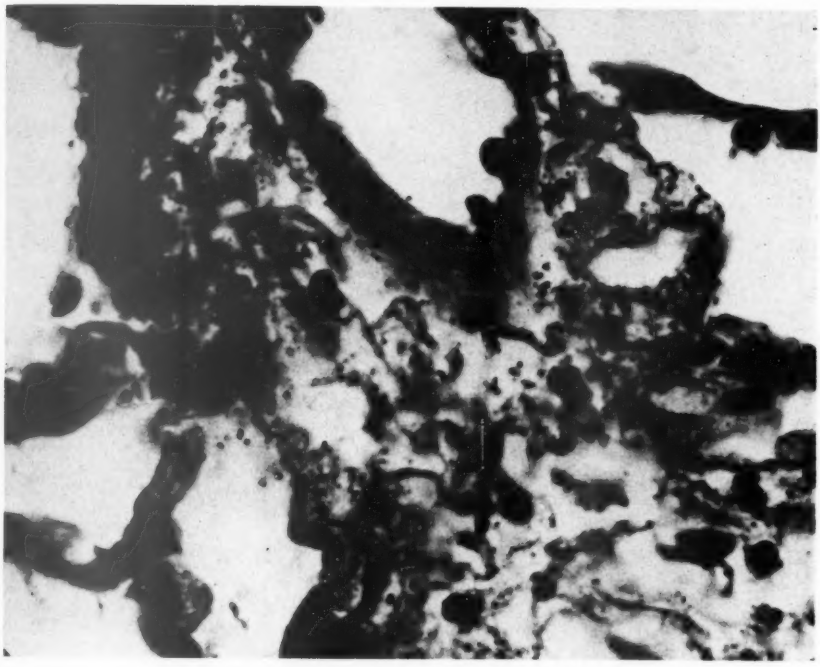
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Section from an excised lung made to "respire" carbon dust for 4 hours at "intrapleural" pressure of ± 4 cm. of Hg and at an intratracheal pressure of ± 8 cm. of water. There is penetration of carbon particles into the edematous stroma. Hematoxylin and eosin stain. × 1150.

FIG. 2. Same section as Figure 1. The tendency of the finely divided particulate matter to cling to connective tissue fibrils is well illustrated. Hematoxylin and eosin stain. × 1150.

1



2

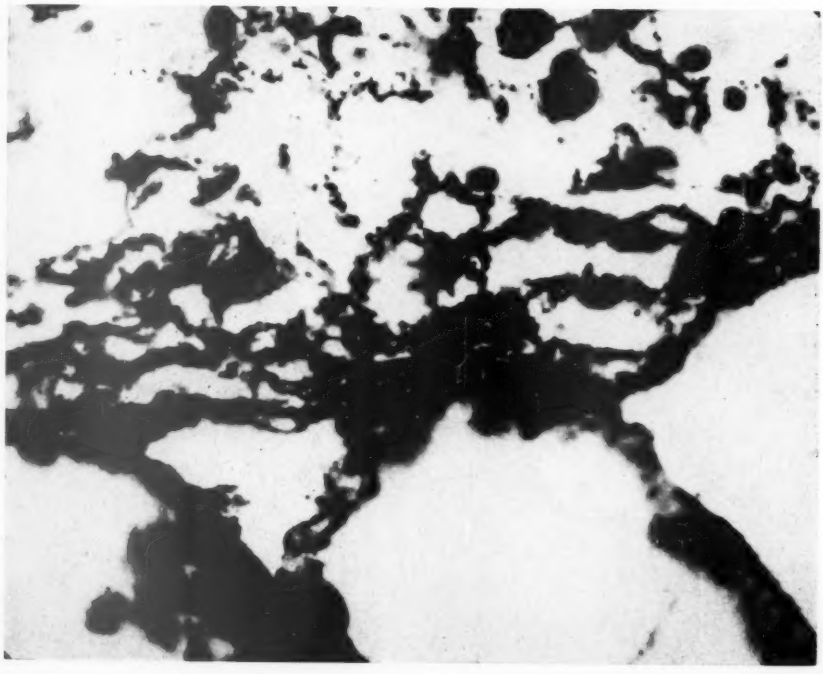
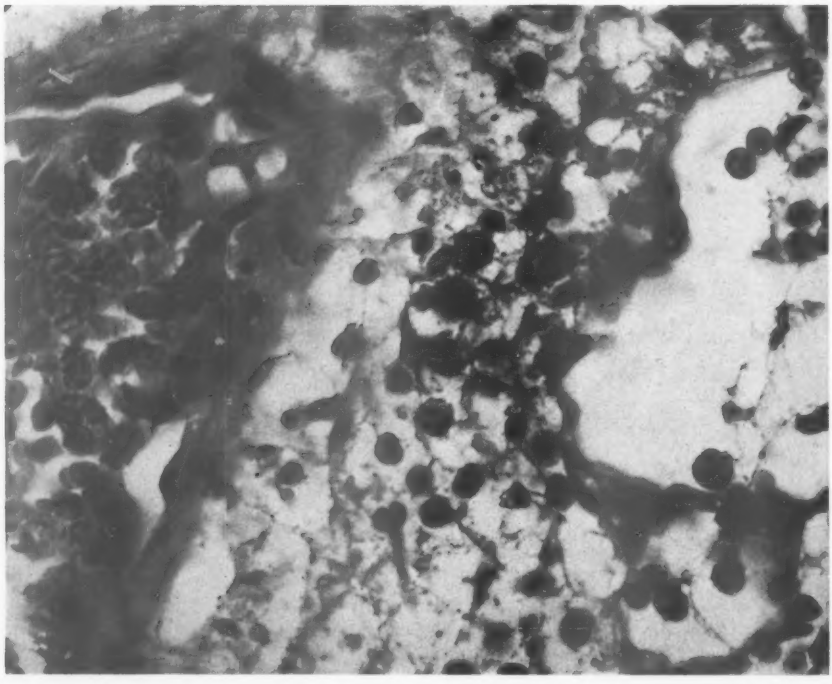
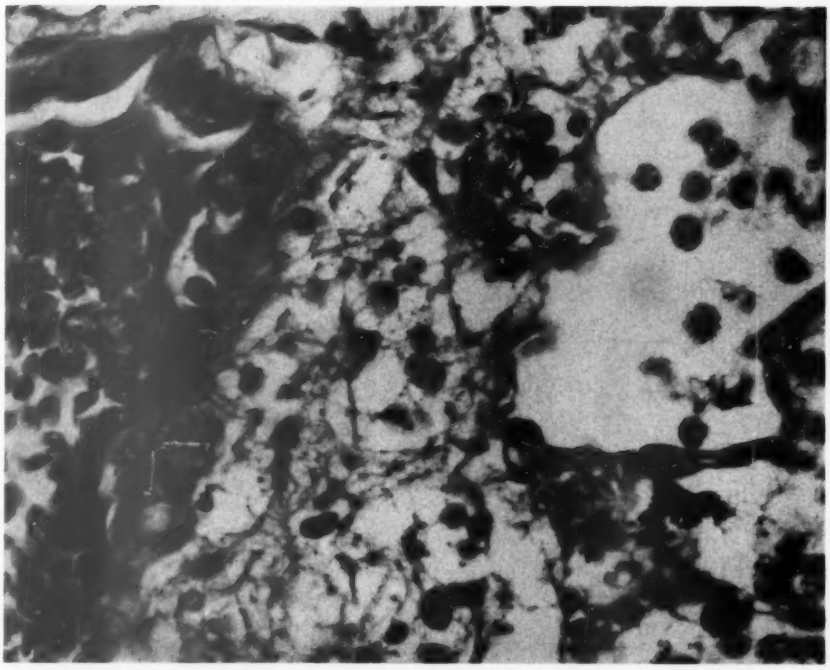


FIG. 3. Section of lung from a rat sacrificed 19 hours following an intratracheal injection of diluted India ink. A focus of pigment penetration is shown at the junction of an alveolus with perivascular stroma. The latter is edematous. The particles, some extremely fine and slightly out of focus, are clinging to fibrils and a few are free in the interstices. Some particles are also upon the surfaces of cells or within their cytoplasm. Hematoxylin and eosin stain. × 1150.

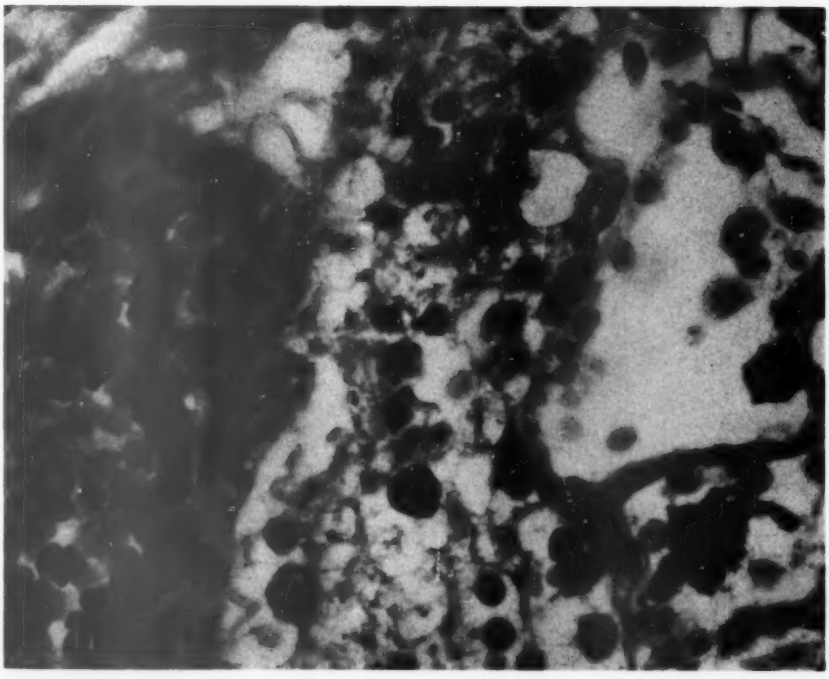
FIG. 4. Section immediately subjacent to that of Figure 3 showing the same area of dust penetration, which is somewhat better defined although scattered minute particles also are found a short distance from the area. Hematoxylin and eosin stain. × 1150.

FIG. 5. Section immediately subjacent to that of Figure 4. The same focus of dust penetration shows particles more compactly distributed giving the area a smudged appearance. A large, blister-like accumulation of edema fluid is present immediately beneath the alveolar lining. A portion of this vesicle is sharply outlined by a fibril which is heavily impregnated with pigment particles. Some particles are found also a short distance away from the focus. Hematoxylin and eosin stain. × 1150.





4



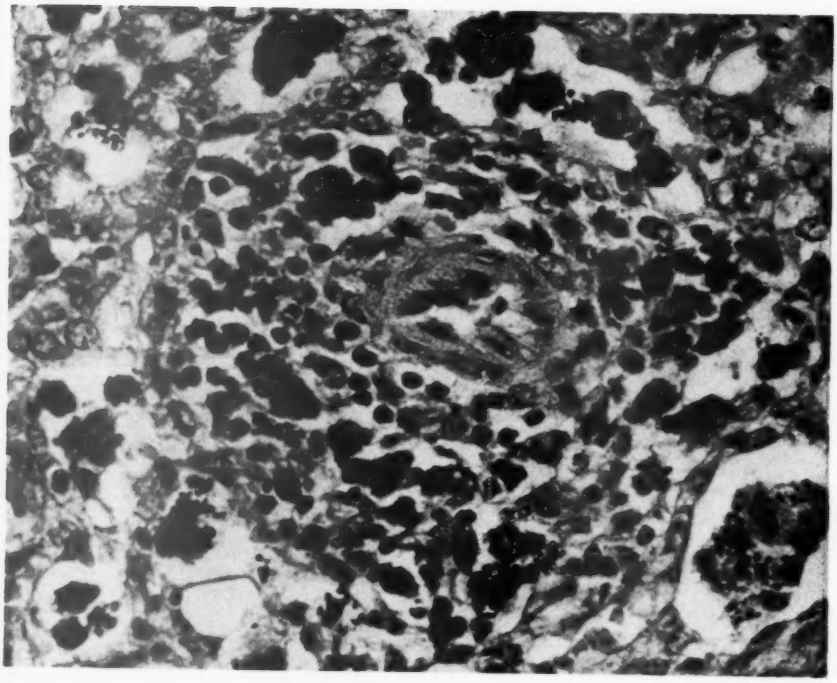
5

FIG. 6. Section of lung from rat sacrificed 4 days following an intratracheal injection of diluted India ink. The perivascular stroma is edematous and replete with cells which resemble those of fixed tissue. Much agglomerated pigment is present. There are no fine particles. Many aggregates are too large and too irregular to represent cells. Other smaller aggregates are obviously extracellular. Some pigment is found in relation to cells. Hematoxylin and eosin stain. × 1150.

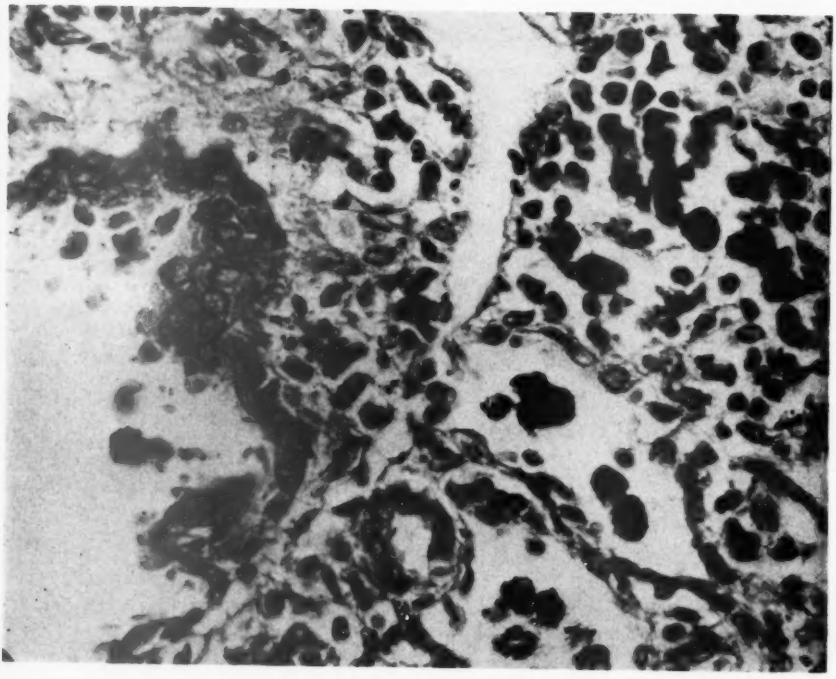
FIG. 7. Section of lung from the same rat as that from which Figure 6 was taken. The edematous peribronchial stroma contains an increased number of connective tissue cells and also pigment aggregates. Finely divided pigment is absent. The pigment masses are extracellular, although a few are attached to the periphery of cells or partially overlie them. There is a lymphatic space in the midportion of the field, to the right of the midline. A number of small, discrete, extracellular aggregates are seen in close relation to the lumen. Hematoxylin and eosin stain. × 1150.

6

7



6



7

STUDIES ON THE TOXICITY OF TYPHUS RICKETTSIAE

II. Pathologic Findings in White Rats and White Mice *

F. Parker, Jr., M.D., and Franklin A. Neva, M.D.†

(From the Mallory Institute of Pathology, Boston City Hospital, and the Department of Microbiology, Harvard School of Public Health, Boston, Mass.)

The toxicity of typhus rickettsiae for white mice is well established,[1-3] but there is relatively little information available concerning associated pathologic lesions. Siegert[4] described microscopic endovascular and perivascular changes found in mice and rats within a few hours after inoculation with rickettsiae, but his note does not contain details regarding specificity of findings, relationship of lesions to time of the animal's death, or size of inoculum. Smadel[5] mentioned only general vascular congestion as the pathologic response to the toxic properties of rickettsiae. Other observers[6] have failed to find specific lesions in selected organs of mice dying of acute rickettsial toxicity.

The first paper of this series[7] described the susceptibility of the white rat to the lethal toxic effect of typhus rickettsiae administered intravenously, and made preliminary note of the prominent gross lesions. It is the purpose of this report to describe the characteristic pathologic lesions in white rats and white mice specifically attributable to the toxic effects of typhus rickettsiae.

Methods

The preparation of rickettsial suspensions from pooled yolk sacs of infected embryonated eggs, their storage, and handling were the same as previously described.[7] Normal yolk sac suspensions for control inoculations were prepared from normal 14-day chick embryos in the same manner as the infected pools, but were stored at approximately −15°C. rather than −70°C. Dilutions of yolk sac suspensions were made with a sterile buffer solution referred to as sucrose PG[8] which contains 0.218 M sucrose; 0.0049 M potassium glutamate; KH_2PO_4, 0.00376 M; and K_2HPO_4, 0.0071 M, at pH 7.0. All yolk sac suspensions were centrifuged at 1000 r.p.m. for 5 minutes to remove particu-

* This work was supported by grants from the Lederle Laboratories Division of the American Cyanamid Company and from the Division of Research Grants of the National Institutes of Health, U.S. Public Health Service (E29C5). Dr. Neva was aided by a Fellowship from the National Foundation for Infantile Paralysis, Inc.

Received for publication, July 22, 1953.

† Now at the Virus Research Laboratory, Municipal Hospital, University of Pittsburgh School of Medicine, Pittsburgh 13, Pa.

late material before inoculation into animals. Heat inactivation of rickettsial suspensions was accomplished at 56°C. for 30 minutes.

Unless specifically noted to the contrary, aliquots of a single pool of the Breinl strain of epidemic typhus rickettsiae *(Rickettsiae prowazeki)* were used throughout. For certain experiments, clearly designated in the text, aliquots of a single pool of the Wilmington strain of murine typhus *(R. mooseri)* were used. The potency of these two pools was checked at intervals by assay of their acute toxic LD_{50} in mice.[9]

Animals used were white male rats 130 to 200 gm. in weight obtained from the Harvard Biological Laboratory ("Hisaw strain") and white Swiss mice of both sexes, 12 to 18 gm. in weight, obtained from the Tumblebrook Farm, Brant Lake, New York. Injection of both rats and mice was made into a tail vein under light ether anesthesia. No recognizable laboratory infection was present in the animals used for this study and no unexplainable spontaneous deaths were noted in the animals used. Rats were fed on sliced raw potato, Purina fox chow pellets, and water *ad libitum;* mice received fox chow pellets and sliced raw potato as their only source of water.

Animals were sacrificed for histologic study by ether anesthesia and subsequent exsanguination. Tissue specimens were obtained immediately (5 minutes or less) after death of the animal. Specimens of intestine were cut in lengths of 3 cm. or less and the lumen of the bowel was gently rinsed with fixative. All tissue specimens were fixed in Zenker's acetic acid (5 per cent) as soon as they were removed. Brains were fixed in formalin.

Paraffin sections of the various organs fixed in Zenker's fluid were stained routinely with Mallory's phloxine and methylene-blue stain.

Paraffin sections of the brains were stained with hematoxylin and eosin and with Nissl's stain.

PATHOLOGIC FINDINGS IN WHITE RATS AFTER A LETHAL TOXIC DOSE OF RICKETTSIAE

Gross Findings

Animals injected with doses of rickettsiae sufficient to produce death within 3 to 6 hours regularly exhibited engorged and dilated visceral blood vessels. The peritoneal surface of the abdominal wall usually was reddened or injected. The liver and spleen appeared dark and congested but, except for an occasional infarct of a portion of the spleen, were otherwise grossly normal. When the intestinal tract was examined it was found that the lower duodenum and jejunum were

usually much darker than other portions of the bowel, and at times these areas of the small bowel appeared hemorrhagic. When the gut presented this appearance, the serosal surface did not appear to be involved but hemorrhage was found on the mucosal surface and the lumen of the gut contained free bloody fluid. Even when hemorrhage was not present, the lumen of the small bowel often contained some yellowish fluid. The intestinal lesions are illustrated in Figures 1 and 2. Other organs such as heart, lungs, kidneys, and brain showed no gross abnormalities and no free fluid was present in body cavities.

Rats injected with somewhat smaller doses of rickettsiae which produced death 6 to 8 hours after injection were less likely to exhibit the gross changes described; and when present, they were less prominent. Rats which were given doses near the toxic LD_{50} range and which succumbed 10 to 18 hours following injection usually showed no definite gross abnormalities.

Microscopic Findings

Significant microscopic changes were found in the intestines, lung, and liver in animals which succumbed within 6 hours after injection.

Intestines. The capillaries of the intestinal mucosa were intensely congested and presented aneurysmal dilatations on the luminal side with rupture and hemorrhages into the lumen. In a rare instance, there was diapedesis of red cells into the mucosal tissue. The mucosal epithelium overlying these congested areas had sloughed off and the surface was covered with red blood cells and amorphous débris (Figs. 3 and 4). The most marked vascular changes, as a rule, were on the side of the intestine opposite the mesenteric attachment.

Lungs. The pulmonary alveolar capillaries contained numerous polymorphonuclear leukocytes, while the alveoli contained no exudate. The small veins were filled with large mononuclear cells and in some vessels the endothelial lining appeared lifted by edema of the underlying tissue.

Liver. The Kupffer cells showed marked phagocytosis of polymorphonuclear leukocytes which appeared intact. This can be seen in Figure 5. The portal areas contained a few lymphocytes and large mononuclear cells.

Brain. Multiple microscopic sections of several brains showed no abnormalities.*

* We are indebted to Dr. Joseph M. Foley, Department of Neurology, Harvard Medical School, Mallory Institute of Pathology, Boston City Hospital, for his examination and report of the brain sections.

PATHOLOGIC FINDINGS IN WHITE MICE AFTER LETHAL TOXIC DOSES OF RICKETTSIAE

After the gross lesions in the intestine of rats had been noted, mice which had died of acute rickettsial toxicity were examined with particular attention to the small bowel. Although congestion of the viscera in general was present, it was apparent that the small intestine was mainly involved. Again, as in the rat, severity of the change was related to the size of dose used and the consequent rapidity of death of the animal. However, the degree of vascular engorgement of the small bowel was never as marked as in rats; only in occasional instances did the lesions appear to be actually hemorrhagic. When the small intestine in the areas involved was opened, the lumen usually contained varying amounts of yellowish fluid and the mucosal surface was reddened. The heart, lungs, brain, and other organs showed no gross abnormalities.

Microscopic Findings

The intestines and livers were the only organs showing significant lesions microscopically in mice that died within 4 hours after injection.

Intestines. The capillaries of the intestinal mucosa were markedly congested but this change was not so severe as in the rats and no herniations into the intestinal lumina or intraluminal hemorrhages occurred.

Liver. The Kupffer cells showed marked phagocytosis of intact polymorphonuclear leukocytes.

DEMONSTRATION OF RICKETTSIAE IN ORGANS

Mice. Rickettsiae have been demonstrated in the liver, lung, and spleen of mice injected with a lethal toxic dose of typhus rickettsiae. This has been accomplished by microscopic examination of impression smears stained with Macchiavello's stain, and also by examination of smears with the fluorescence microscope for specific staining of rickettsiae by the method of fluorescent-labelled antiserum.[10] Faint specific staining with fluorescent antiserum was noted only in the spleens and livers of mice which had been injected with lethal toxic doses of typhus rickettsiae. Results, however, have not been consistently successful, and the numbers of rickettsiae have been few by either of the methods employed; consequently the question of localization of rickettsiae in the animal following a lethal toxic dose was not pursued further.

Rats. In experiments with white rats a few rickettsiae were seen in impression smears of liver, lungs, and spleen stained with Macchiavello's stain, after a lethal toxic dose had been given. No rickettsiae

were observed in smears from congested or hemorrhagic small intestine from either mice or rats.

PATHOLOGIC FINDINGS IN WHITE RATS AFTER A SUBLETHAL TOXIC DOSE OF RICKETTSIAE

The microscopic findings in white rats dying of acute rickettsial toxicity suggested the possibility that further passage of time before death of the animal might allow for the development of more prominent lesions. Several experiments were performed, therefore, in which groups of white rats were injected each with approximately one LD_{50} or slightly less than one LD_{50} toxic dose of epidemic typhus rickettsiae; the animals were sacrificed after varying intervals of time. It should be emphasized that white rats and mice which receive *epidemic* typhus rickettsiae intravenously either die in 18 hours from the toxic effects, or they survive without subsequent evidence of disease. By contrast, sublethal toxic doses of *murine* typhus rickettsiae establish an infection in white rats and mice which may be fatal 3 to 8 days after inoculation.

Gross Findings

There were no gross lesions in any of the animals in this group.

Microscopic Findings

There were no intestinal lesions at 12 hours. The changes in the lungs and liver were similar to those in the animals dying an acute death. At 24 hours the principal lesions were confined to the lungs and liver.

Lungs at 24 Hours. The alveolar capillaries contained numerous polymorphonuclear leukocytes but the veins did not contain the numerous mononuclear cells noted earlier.

Liver at 24 Hours. The Kupffer cells contained numerous polymorphonuclear leukocytes, some of which appeared degenerated or necrotic, and in addition an occasional red cell and lymphocyte. In the lumina of the sinusoids there were a few focal collections of large mononuclear cells and an occasional polymorphonuclear leukocyte. A rare liver cell appeared to have degenerated or to be undergoing necrobiosis. The portal areas contained a few large mononuclear cells, lymphocytes, and plasma cells.

Lungs at 48 Hours. The alveolar capillaries of the lungs contained few polymorphonuclear leukocytes in comparison to the animals killed earlier.

Liver at 48 Hours. In addition to the phagocytic Kupffer cells noted

24 hours earlier, at 48 hours there were occasional necrotic liver cells invaded by large mononuclear cells and polymorphonuclear leukocytes. Figure 6 shows such an area. The portal areas were infiltrated with numerous large mononuclear cells and lymphocytes.

Lungs at 72 Hours. The changes in the lungs at 72 hours were negligible.

Liver at 72 Hours. There were numerous and extensive foci of necrosis of liver cells at 72 hours. These lesions were scattered in a haphazard fashion throughout the lobules. The necrotic hepatic cells were invaded by large mononuclear cells and polymorphonuclear leukocytes. These lesions are illustrated in Figures 7 and 8. In addition to focal necrosis, there were lesions in the sinusoids composed of focal collections of large mononuclear cells, a few polymorphonuclear leukocytes, and sometimes red blood cells and fibrin (Fig. 9). Such lesions were apparently not associated or connected with necrotic liver cells. The Kupffer cells were swollen and many contained living and dead polymorphonuclear leukocytes, red blood cells, lymphocytes, and occasionally a necrotic liver cell. Numerous Kupffer cells were in mitosis. The portal areas were infiltrated with many large mononuclear cells, lymphocytes and, in places, with mast cells. In addition, both in the portal areas and in the sinusoids there were numerous immature mononuclear cells, some of which were in mitosis.

Liver at 96 Hours. The hepatic lesions were less marked and less extensive at 96 hours. An occasional lesion appeared to be healing since it was surrounded by a clear space as though shrunken away from the surrounding tissues. Mitotic figures were present in the liver cells surrounding such lesions, as shown in Figure 10.

Liver at 5 Days. The lesions in the liver had disappeared at 5 days, with no scarring or other evidence of their existence. The only changes present were a few small, focal collections of mononuclear cells in the sinusoids and a slight infiltration of the portal areas with large mononuclear cells and lymphocytes. A few Kupffer cells contained necrotic cells. Mitotic figures were present in the liver cells but were few in number.

At 7 days and 12 days there were no lesions present.

Lesions in organs other than those described occurred, but were not sufficiently constant or distinctive to be considered pathognomonic. The *heart* in several animals showed necrosis of single muscle fibers. In one animal this was extensive. However, such a finding was not common. A more constant finding was an infiltration of large mononuclear cells in the interstitial tissue of the myocardium and beneath

the endocardium. The *spleen* often contained many phagocytes filled with red blood cells and, in some instances, phagocytes containing hemosiderin were numerous. Since such changes are seen frequently in other conditions, they were not considered pathognomonic. In the *kidneys* of several animals there were focal collections of large mononuclear cells in the interstitial tissue. The *adrenal glands, lymph nodes, bone marrow,* and *brain* showed no definite lesions.

Further Experiments with Sublethal Toxic Doses

In an effort to determine whether the hepatic lesions which develop following a sublethal dose are related to multiplication of rickettsiae in the liver, the following experiment was performed.

Eleven white rats, weighing 160 to 175 gm., were each injected intravenously with approximately one half of an LD_{50} toxic dose of epidemic typhus rickettsiae. Rats were sacrificed in pairs by ether anesthesia and exsanguination from the heart at intervals of 6 hours and of 1, 3, 5, and 7 days; the one remaining animal was examined 12 days after injection; 1.3 to 1.8 gm. portions of liver were removed from each animal; the two portions were pooled and a 25 per cent wet weight suspension was prepared in sterile sucrose PG solution[8] by grinding with alundum. The suspensions were quickly frozen in an alcohol-dry ice bath and stored at −70°C. Pieces of liver were removed also for tissue sections and impression smears. The frozen liver suspensions were subsequently thawed and titrated for viable rickettsiae by inoculation of serial 1.5 log dilutions intra-abdominally into cotton rats, by the standard method of this laboratory.[11] * The results of the titrations are shown in Table I.

The numbers of rickettsiae dropped sharply so that by 3 days after injection no viable organisms were detected at the lowest dilution tested. The very few organisms demonstrated on the fifth day probably were attributable to sampling variation by the method used, rather than to significant increase due to multiplication. Impression smears of livers stained with Macchiavello's stain were negative for rickettsiae in all of the animals sacrificed, even as early as 6 hours after injection with a sublethal toxic dose. Table I also shows the degree of hepatic lesions found microscopically in comparison with the results of rickettsial titration. There was no evidence of rickettsial

* The titration in cotton rats was done through the generous cooperation of Dr. E. S. Murray and Mrs. Doris Simon of the Department of Microbiology, Harvard School of Public Health.

multiplication in the livers of the white rats at the time that the hepatic lesions were most prominent.

ADDITIONAL EVIDENCE FOR RELATIONSHIP OF HEPATIC LESIONS IN WHITE RATS TO TOXIC EFFECT OF TYPHUS RICKETTSIAE

One of the features of acute rickettsial toxic death in both white mice and white rats is that the effect can be neutralized with specific antiserum. This was also found to be true of hepatic lesions following sublethal toxic doses of rickettsiae. Two white rats injected with a sub-

TABLE I

Comparison of Hepatic Lesions with Numbers of Viable Rickettsiae in the Liver Following a Sublethal Toxic Dose of Epidemic Typhus to White Rats

Time	6 hours	24 hours	3 days		5 days	7 days	12 days
Rat number	1 2	3 4	5	6	7 8	9 10	11
Degree* of hepatic lesions	o o	+ +	++++	++++	o o	o o	o
Number of ID_{50}'s per gram of liver†	1,264,000	104,000	<12		80	<12	<12

* Estimated on the basis that o = no definite changes and ++++ = the most prominent lesions seen.

† This value represents the number of 50 per cent immunizing doses for cotton rats by titration of pooled livers from 2 rats with exception of rat 11 which was a single specimen.

lethal toxic dose plus anti-epidemic typhus rabbit serum failed to show any hepatic lesions when sacrificed 3 days later.

Livers of white rats injected intravenously with murine typhus rickettsiae also were studied. In contrast to epidemic typhus rickettsiae, murine typhus rickettsiae in appropriate concentrations produce a lethal infection in white rats 3 to 5 days after injection. In our tests such doses were used; animals were sacrificed at periods of 26, 53, 72, and 96 hours after inoculation for histologic study of the liver and impression smears of the liver were stained with Macchiavello's stain and examined for rickettsiae. In every instance the livers of murine-infected rats showed abundant rickettsiae by smear.

Microscopic Findings

Our chief interest was to compare the microscopic lesions in the livers of this series of animals with those seen in the rats injected with epidemic typhus rickettsiae. The animals were killed at the various periods following injection, as indicated.

Liver at 24 Hours. The portal areas were infiltrated with a few

lymphocytes and large mononuclear cells at 24 hours. There was an occasional focal collection of a few lymphocytes and large mononuclear cells in the sinusoids. A rare Kupffer cell contained a polymorphonuclear leukocyte or lymphocyte.

Liver at 48 Hours. At 48 hours the Kupffer cells were swollen and some were necrotic. Many had phagocytized polymorphonuclear leukocytes and red blood cells. Mitotic figures in the Kupffer cells were common. These changes can be seen in Figure 11. There were acute focal lesions in the sinusoids, composed of polymorphonuclear leukocytes and fibrin and occasionally a necrotic liver cell. Single necrotic liver cells also were found apart from these lesions. The portal areas were infiltrated with large mononuclear and immature mononuclear cells.

Liver at 72 Hours. At 72 hours the changes were similar to those seen at 48 hours.

Liver at 96 Hours. The changes at 96 hours were similar to those in preceding animals, but the focal lesions were more prominent and single necrotic liver cells more numerous.

HEPATIC LESIONS IN WHITE MICE AFTER SUBLETHAL TOXIC DOSES OF RICKETTSIAE

The occurrence of hepatic lesions following sublethal toxic doses of rickettsiae was also sought in white mice. This was of interest since there is considerable evidence[7,12] that the manifestations of rickettsial toxicity in white rats and white mice are quite similar. Mice were injected with a maximum sublethal toxic dose of epidemic typhus rickettsiae and then sacrificed in pairs after 1 and 3 days. None of these animals showed gross abnormalities.

Microscopic Findings

Liver at 24 Hours. The Kupffer cells were swollen at 24 hours and some contained living and necrotic polymorphonuclear leukocytes. A very rare necrotic liver cell was seen. The portal areas were infiltrated with a few large mononuclear cells, lymphocytes, and plasma cells. A few small focal collections of large mononuclear cells and polymorphonuclear leukocytes were present in the sinusoids.

Liver at 72 Hours. There were scattered foci of necrosis of liver cells with an infiltration in such areas of numerous polymorphonuclear leukocytes, some of which were necrotic, and also large mononuclear cells (Fig. 12). The Kupffer cells were swollen and a considerable

number contained polymorphonuclear leukocytes. Mitotic figures were present in Kupffer cells. The portal areas contained a few large mononuclear cells, lymphocytes, and plasma cells.

CONTROL STUDIES

In addition to the use of antiserum to prevent development of hepatic lesions and the study of hepatic lesions in rats infected with murine typhus, further attempts were made to establish the specificity of the lesions related to rickettsial toxicity. Both rats and mice which had been injected with normal yolk sac suspensions as well as with suspensions of heat-killed rickettsiae were studied to exclude nonspecific lesions. Specimens for histologic study were taken several hours after injection and 3 days after injection. No gross abnormalities were noted in these control animals and no specific microscopic lesions were found in any of the animals.

DISCUSSION

The principal pathologic manifestations of the toxic effects of typhus rickettsiae were of two main types: the gross lesions observed at death, and the microscopic lesions which resulted from sublethal doses. Hemorrhage and intense congestion of the intestinal mucosa were the chief lesions in those animals which died within a few hours. In contrast, the microscopic hepatic lesions were most prominent on the third day following a sublethal dose. Yet the evidence which has been presented indicates that both the intestinal and hepatic lesions were related to the toxic effect of viable typhus rickettsiae.

The changes noted in the lungs and livers within a few hours after injection with lethal doses of rickettsiae were of a non-specific nature. The intestinal lesion, however, was unique—the midportion of the bowel was the consistent site of major involvement. Hemorrhage involving tips of mucosal villi is not a lesion of the type which occurs in agonal and anoxic states. The possible relationship of the site of these changes with intestinal digestive glands was not investigated.

There was no obvious evidence of acute loss of fluid or plasma into interstitial tissues except for the intraluminal escape of blood in the small bowel. This may be a factor to consider in relation to the physiologic effects associated with acute rickettsial toxicity in mice and rats, a subject which is dealt with in a separate communication.[12]

Although pathologic changes resulting from the toxic effect of typhus rickettsiae could be demonstrated in both white rats and white mice, the latter animals showed minimal intestinal lesions after lethal doses.

A satisfactory explanation for this difference has not been found. Both rats and mice, however, exhibited hepatic lesions of identical types following sublethal toxic doses of rickettsiae.

It is of interest that the hepatic lesions reached their peak in 3 days. These focal lesions were minimal, although present 24 hours following a sublethal toxic dose, but became progressively more prominent by 3 days and had entirely healed by 5 days after inoculation. For several reasons these hepatic lesions were attributed specifically to the toxic effects rather than to multiplication of the rickettsiae. First, rickettsial organisms were not demonstrated in smears of the livers. Second, titration of the liver suspension for viable rickettsiae showed that while the lesions became increasingly prominent, there was a progressive and rapid decrease in numbers of rickettsiae. The type of change—focal necrosis of liver cells—remains consistent with a toxic etiology. Finally, the pathologic changes in the livers of rats in which extensive multiplication of murine typhus rickettsiae was occurring showed notable differences from the hepatic lesions in animals given a sublethal toxic dose.

The main pathologic feature of the livers infected with murine typhus was that of diffuse reaction and injury to the Kupffer cells, but some necrosis of single liver cells did occur. The toxic damage after sublethal toxic doses of epidemic typhus rickettsiae appeared chiefly as extensive focal necrosis of liver cells, yet some damage to Kupffer cells also was apparent. So, although the pathologic appearance of murine infection and toxic damage were easily differentiated, they did show some features in common.

In our study no evidence was found of perivascular lesions in the brain such as Siegert[4] attributed to the toxic effect of typhus rickettsiae. Furthermore, our findings of lesions in the liver due to acute rickettsial toxicity were at variance with Siegert's description. He made no mention in his report of the intestinal lesions which we have described, nor did he present convincing evidence that the inocula contained fully viable rickettsiae. The acute toxicity of rickettsiae with which we are concerned in this report is highly labile and intimately dependent upon the viability of the organisms.

Wolbach, Todd, and Palfrey,[13] in describing the pathology of epidemic typhus *infection,* found certain lesions similar to those which we believe are related to rickettsial *toxic* effect. They found that both human and guinea-pig livers showed large swollen Kupffer cells, often containing red and white cell débris. Also in man they found occasional necrotic liver cells, and in both man and the guinea-pig discrete

lesions of proliferating endothelial cells were noted. The main feature of epidemic typhus infection described by Wolbach *et al.*, however, is primarily an endothelial vascular involvement; this was not a conspicuous lesion in our study of toxic damage produced by epidemic typhus. It should be emphasized that the toxic phenomenon is observed only when enormous numbers of viable typhus rickettsiae are injected into animals. Comparisons of pathologic changes, therefore, between the experimentally induced toxic phenomenon in animals and the naturally occurring infection in man may be misleading.

Acute toxic death in mice resulting from intravenous inoculations of large doses of the lymphogranuloma-psittacosis group of viruses[14,15] and influenza viruses[16] has been described. As with the phenomenon of rickettsial toxicity, the effects of viral toxins appear to be intimately related to the infective virus particles. Rake and Jones[14] reported that lesions of the liver were minimal in mice injected with lymphogranuloma venereum virus when death occurred before 9 hours after injection, but very prominent foci of hepatic necrosis were found in later toxic deaths. Focal necrosis of the liver also was present in mice dying of toxic doses of meningopneumonitis virus. But no pathologic changes were found in association with toxicity of mouse pneumonitis virus when death occurred within 4 hours after injection. The microscopic hepatic lesions reported by these authors presented many similarities to the rickettsial toxic lesions. No intestinal lesions were reported by Rake and Jones although hemorrhage was noted in other organs.

Focal necrosis in the liver was described also by Henle and Henle[16] in their studies on the toxicity of influenza viruses. With these agents the earliest deaths in mice occurred about 8 hours after intravenous injection and with smaller doses the animals died in 24 to 48 hours. In addition to striking hepatic necrosis, the Henles also mentioned that with toxic death from some strains of influenza A, intestinal blood vessels were engorged and the gut contained bloody mucous material. This description appears to correspond very closely with the type of intestinal lesions we have noted with acute rickettsial toxicity in white rats. Moreover, the microscopic lesions in the liver reported by the Henles are similar to our findings.

Focal hepatic lesions and hemorrhages in various organs, including the intestine, are among the more prominent pathologic findings in animals given various bacterial endotoxins.[17-19] Functional damage to the livers of rats, as evidenced by decrease in liver glycogen, has been reported following the administration of bacterial endotoxins[20] as well as heat-killed rickettsiae.[21] This suggests a possible relationship of the

pathologic effects of bacterial endotoxins to the toxic properties of certain viruses and rickettsiae.

Summary

In white rats toxic doses of typhus rickettsiae which caused death within 6 hours produced mucosal hemorrhage and intense congestion in the midportion of the small bowel. In addition, polymorphonuclear leukocytes had accumulated in the alveolar capillaries of the lung and had been extensively phagocytized by the Kupffer cells of the liver.

In white mice toxic doses which caused death within 4 hours produced only vascular engorgement of the small intestine grossly but no hemorrhage into the lumen. The only definite microscopic lesions in mice were in the liver and were similar to those described in white rats.

Sublethal toxic doses of rickettsiae produced prominent microscopic lesions in the livers of both rats and mice. These consisted of focal necrosis of hepatic cells which became progressively more prominent up to the third day after injection, and had healed entirely by the fifth day. The pathologic changes were attributed specifically to the toxic effects of the rickettsiae rather than to their multiplication.

The toxic lesions produced by rickettsiae were compared with the toxic changes in mice caused by certain viruses.

The photomicrographs were taken by Mr. Leo Goodman, Mallory Institute of Pathology, Boston City Hospital. We are indebted to Dr. J. C. Snyder for his helpful assistance and advice in the preparation of this paper.

REFERENCES

1. Gildemeister, E., and Haagen, E. Fleckfieberstudien. I. Mitteilung: Nachweis eines Toxins in Rickettsien-Eikulturen *(Rickettsia mooseri)*. *Deutsche med. Wchnschr.*, 1940, **66**, 878–880.
2. Bengtson, I. A., Topping, N. H., and Henderson, R. G. Studies of typhus fever. Epidemic typhus: Demonstration of a substance lethal for mice in the yolk sac of eggs infected with *Rickettsia prowazeki*. *Nat. Inst. Health Bull.*, 1945, **183**, 25–29.
3. Hamilton, H. L. Specificity of the toxic factors associated with the epidemic and the murine strains of typhus rickettsiae. *Am. J. Trop. Med.*, 1945, **25**, 391–395.
4. Siegert, R. Das Rickettsientoxin als Ursache histopathologischer Organveränderungen. *Ztschr. f. Hyg. u. Infectionskr.*, 1948, **128**, 477–485.
5. Smadel, J. E. In: Rivers, T. M. (ed.) Viral and Rickettsial Infections of Man. J. B. Lippincott Co., Philadelphia, 1952, ed. 2, pp. 89–91.
6. Snyder, J. C., and Adams, R. D. Personal communication.
7. Neva, F. A., and Snyder, J. C. Studies on the toxicity of typhus rickettsiae. I. Susceptibility of the white rat, with a note on pathologic changes. *J. Infect. Dis.*, 1952, **91**, 72–78.

8. Bovarnick, M. R., Miller, J. C., and Snyder, J. C. The influence of certain salts, amino acids, sugars, and proteins on the stability of rickettsiae. *J. Bact.*, 1950, 59, 509–522.
9. Henderson, R. G. Studies of typhus fever. Notes on the mouse test with typhus vaccine. *Nat. Inst. Health Bull.*, 1945, 183, 33–35.
10. Coons, A. H., Snyder, J. C., Cheever, F. S., and Murray, E. S. Localization of antigens in tissue cells. IV. Antigens of rickettsiae and mumps virus. *J. Exper. Med.*, 1950, 91, 31–38.
11. Snyder, J. C., Murray, E. S., and Fuller, H. S. In preparation.
12. Neva, F. A., and Snyder, J. C. In preparation.
13. Wolbach, S. B., Todd, J. L., and Palfrey, F. W. The Etiology and Pathology of Typhus. Harvard University Press, Cambridge, 1922, pp. 152–198.
14. Rake, G., and Jones, H. P. Studies on lymphogranuloma venereum. II. The association of specific toxins with agents of the lymphogranuloma-psittacosis group. *J. Exper. Med.*, 1944, 79, 463–486.
15. Manire, G. P., and Meyer, K. F. The toxins of the psittacosis-lymphogranuloma group of agents. I. The toxicity of various members of the psittacosis-lymphogranuloma venereum group. *J. Infect. Dis.*, 1950, 86, 226–232.
16. Henle, W., and Henle, G. Studies on the toxicity of influenza viruses. II. The effect of intra-abdominal and intravenous injection of influenza viruses. *J. Exper. Med.*, 1946, 84, 639–660.
17. Dennis, E. W. Toxicity of acid-soluble typhoid toxin for laboratory animals. *Proc. Soc. Exper. Biol. & Med.*, 1939, 42, 553–554.
18. Morgan, H. R. Pathologic changes produced in rabbits by a toxic somatic antigen derived from *Eberthella typhosa*. *Am. J. Path.*, 1943, 19, 135–145.
19. Burrows, W. Endotoxins. *Ann. Rev. Microbiol.*, 1951, 5, 181–196.
20. Kun, E., and Abood, L. G. Mechanism of inhibition of glycogen synthesis by endotoxins of *Salmonella aertrycke* and type I meningococcus. *Proc. Soc. Exper. Biol. & Med.*, 1949, 71, 362–364.
21. Olitzki, L., Czaczkes, J. W., and Kuzenok, A. Endotoxic factors of *Rickettsia prowazeki* and their immunological relationship to the endotoxins of other gram negative microorganisms. *J. Immunol.*, 1946, 53, 365–370.

LEGENDS FOR FIGURES

All microscopic sections are from Zenker's-fixed tissue stained with Mallory's phloxine and methylene blue stain.

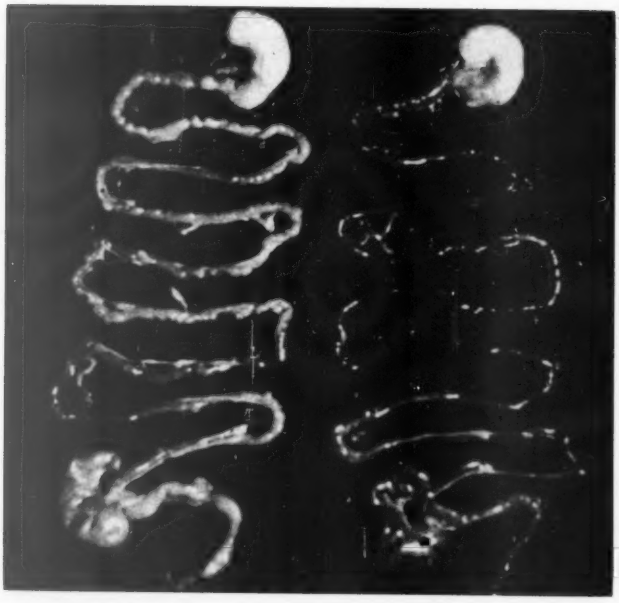
FIG. 1. Gross appearance of gastro-intestinal tract of a rat dying an acute toxic death. The small intestine is markedly congested and edematous compared to the control.

FIG. 2. A segment of the small intestine from the same animal as in Figure 1, compared with a normal control.

VOLUNTARY

XU

1



Control Test

2

Control

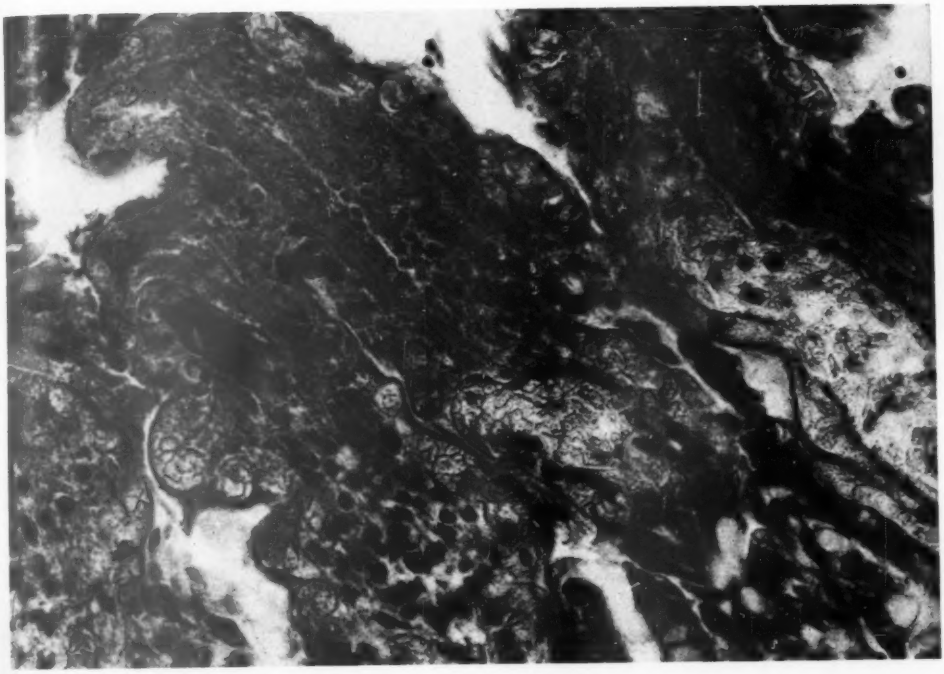
Test

FIG. 3. Small intestine of a rat dying an acute toxic death. This photomicrograph shows marked congestion of capillaries with herniation into the intestinal lumen which contains amorphous débris. × 300.

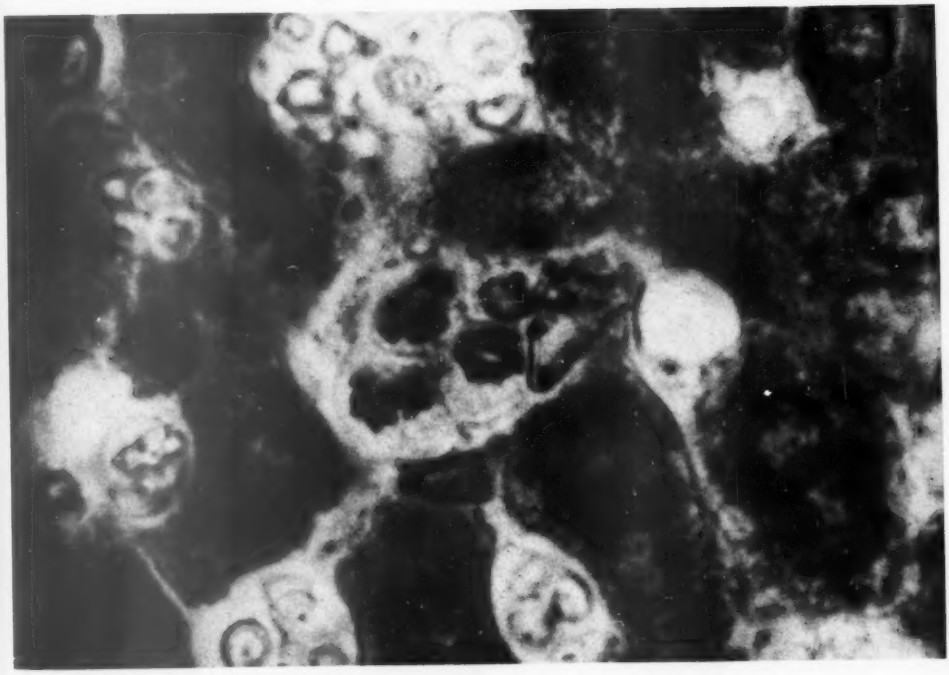
FIG. 4. Same lesion as in Figure 3, showing marked dilatation of mucosal capillaries. × 685.

FIG. 5. Liver from a rat dying an acute toxic death. A phagocytic Kupffer cell contains three polymorphonuclear leukocytes. × 1880.

3



4

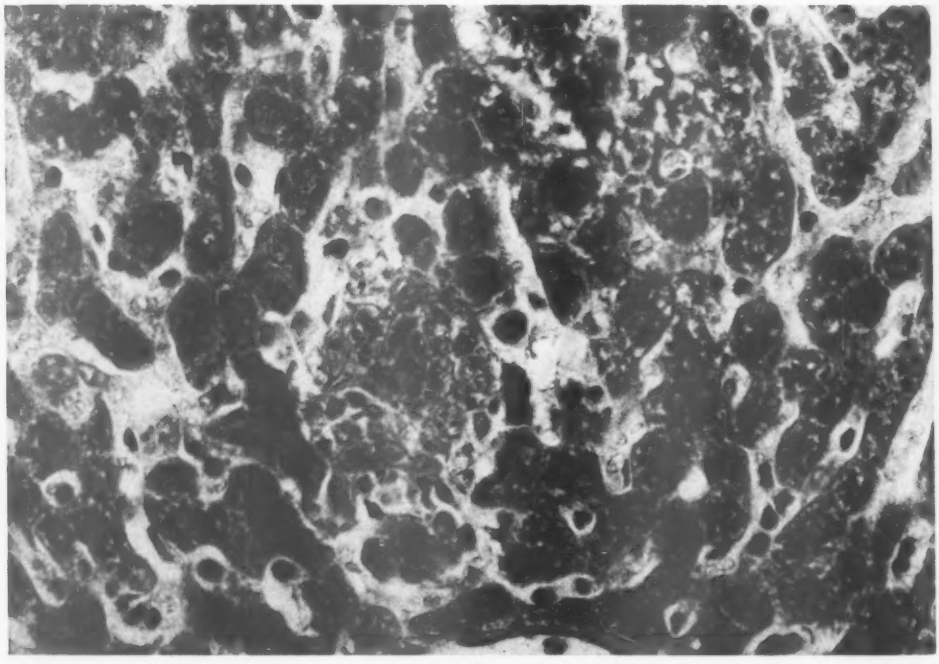


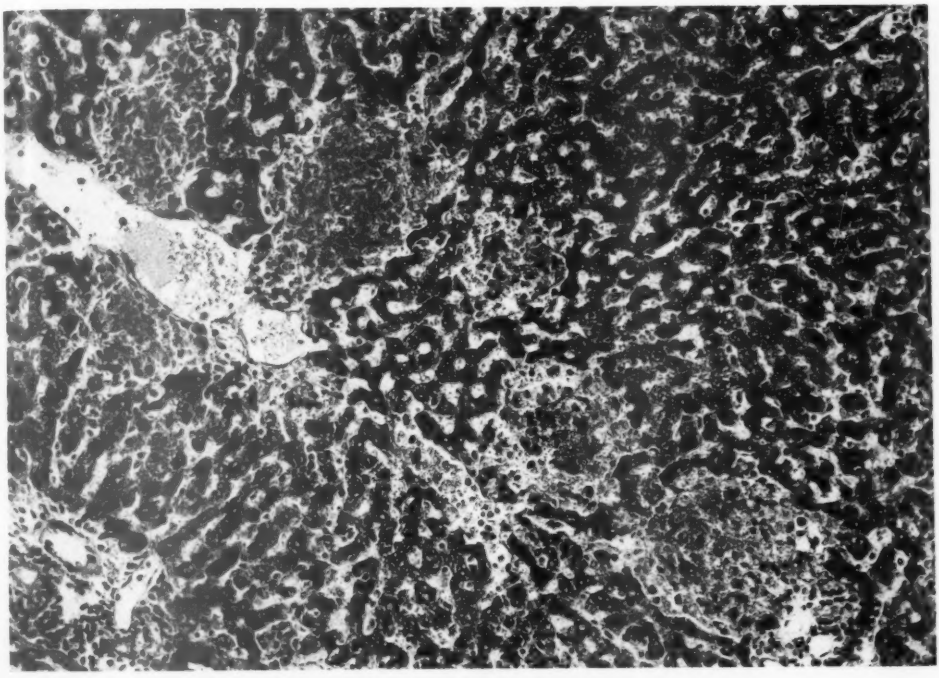
5

FIG. 6. Liver from a rat sacrificed 48 hours after a sublethal dose. There is focal necrosis of a few liver cells with infiltrating large mononuclear cells. × 685.

FIG. 7. Liver from a rat sacrificed 72 hours after a sublethal dose. Several foci of necrosis are shown. × 220.

FIG. 8. Higher power of focal lesions from the same animal as used for Figure 7. There are necrotic liver cells and an accumulation of large mononuclear cells. × 730.





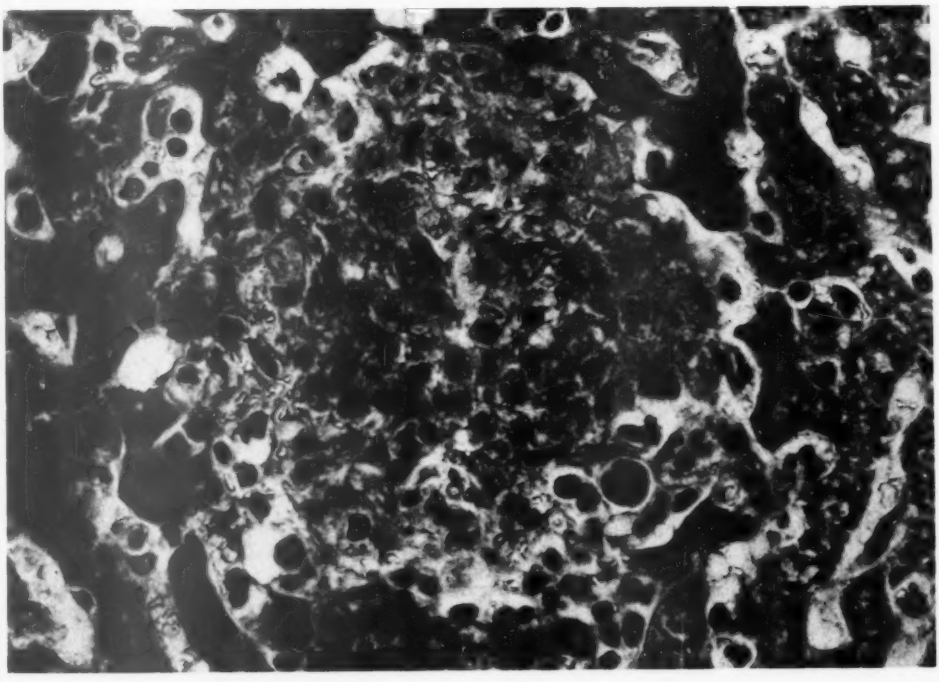
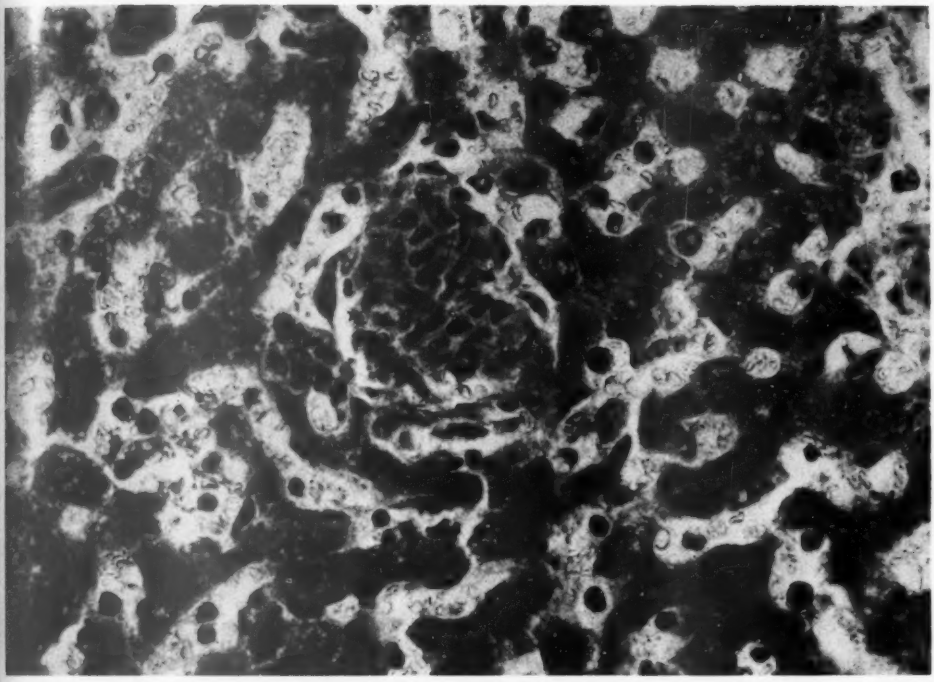


FIG. 9. Liver from the same animal as used for Figures 7 and 8. There is a focal accumulation of large mononuclear cells in a sinusoid, apparently not associated with necrosis of liver cells. × 730.

FIG. 10. Liver from a rat sacrificed 96 hours after a sublethal dose. A focal collection of large mononuclear cells with a clear space around them is seen in a sinusoid. One mitotic figure appears in an adjacent liver cell. × 730.

VOLUME
MARVEL



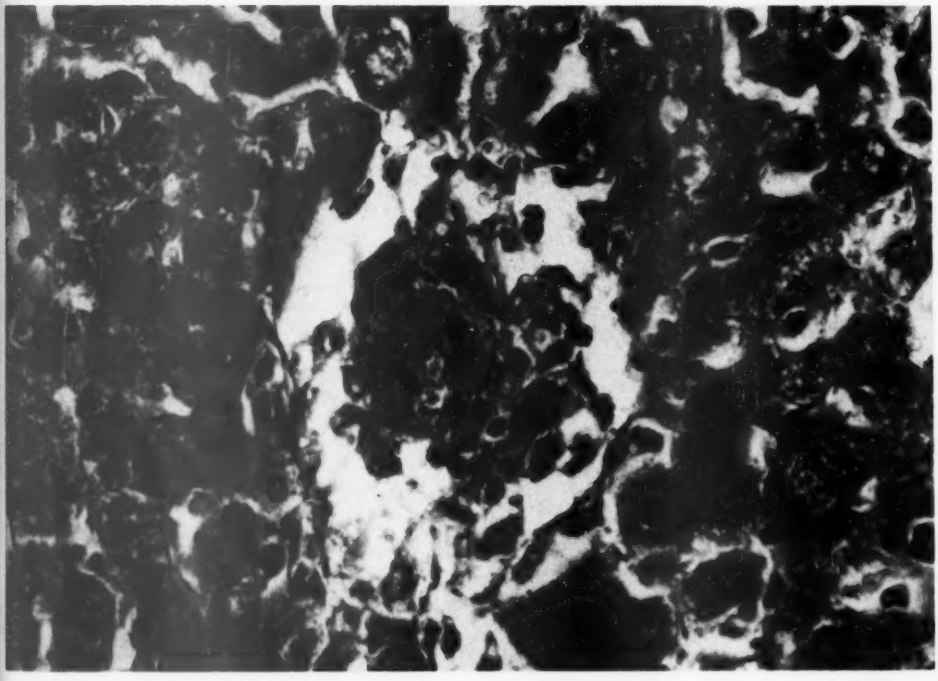
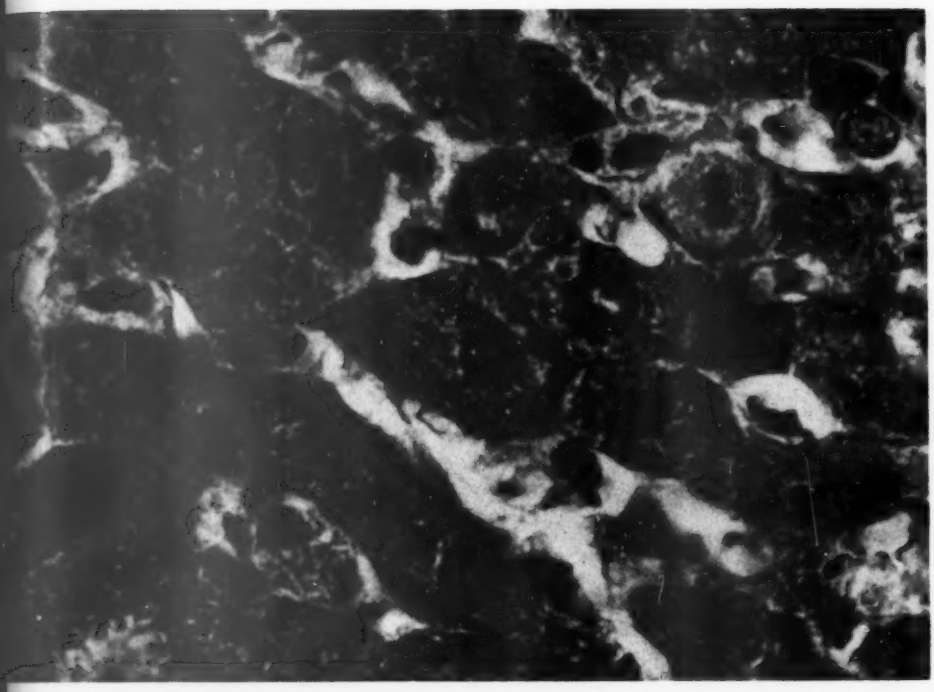


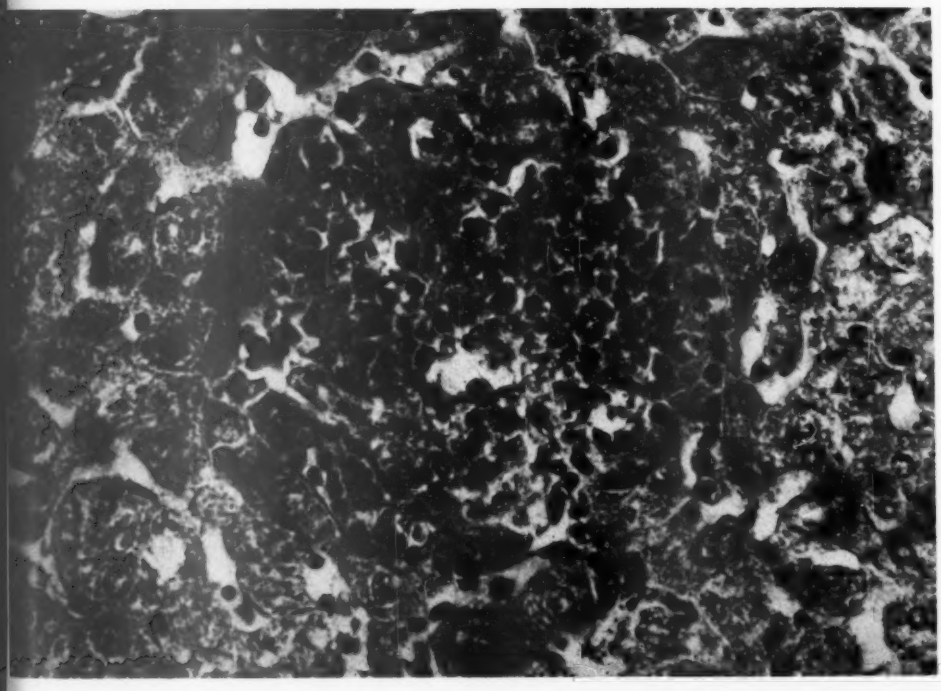
FIG. 11. Liver from a rat sacrificed 48 hours after infection with the murine strain. Swollen Kupffer cells are seen, two of which are in mitosis. × 1375.

FIG. 12. Liver from a mouse sacrificed 72 hours after a sublethal toxic dose of rickettsiae. A focus of necrosis of liver cells is infiltrated with numerous polymorphonuclear leukocytes. × 730.

VOL 2 NO 4 MAR APR 54

XU





THE RESORPTION OF AMYLOID UNDER EXPERIMENTAL CONDITIONS *

GOETZ W. RICHTER, M.D.†

(From the Department of Pathology, the New York Hospital-Cornell Medical Center, New York, N.Y.)

A number of clinical observations have indicated that naturally occurring amyloidosis may regress,[1-3] and several investigators have presented evidence suggesting that regression of amyloidosis has occurred under experimental conditions.[4-7] The previous experimental work usually has involved a comparison of amyloidosis in different animals or in different tissues or paired organs, and no systematic studies have been recorded thus far in which the process of resorption has been demonstrated conclusively through repeated microscopic study of the same organ. The present report provides conclusive evidence that amyloid deposits in the spleens of rabbits, produced by treatment with sodium ribonucleate, were resorbed after cessation of treatment. The morphologic details of the amyloidosis that was produced and of the resorptive process that led to a complete or partial disappearance of amyloid from the spleen, will be given in this paper.

EXPERIMENTAL PROCEDURES

Amyloidosis of the spleen and kidneys, and occasionally of lymph nodes and liver also, was produced in rabbits by injecting sterile solutions of 5 per cent sodium ribonucleate five times a week during periods lasting up to 6 months.

Hybrid rabbits of both sexes, weighing approximately 2000 gm., were used. They were fed Rockland pellets and given water *ad libitum*. The properties and method of preparation of the solutions of ribonucleate have been described in detail in a previous report.[8] The material was a highly purified mixture of yeast nucleotides, and was free of protein.

Laparotomies were performed in a number of rabbits that had been treated for several months in order to obtain portions of their spleens for histologic examination. For this purpose the animals were anesthetized with nembutal and ether, and their spleens exposed aseptically through upper abdominal incisions. Following ligation of branches

* This investigation was supported by a research grant from the National Institutes of Health, United States Public Health Service.

Received for publication, July 8, 1953.

† Lewis Cass Ledyard, Jr., Fellow of the Society of the New York Hospital.

of the splenic artery, approximately one fourth to one third of each spleen was tied off by means of catgut or silk ligatures, and then resected. The incisions were closed with silk. All of these animals recovered promptly from the operation. They were killed and necropsied several weeks later, as will be indicated.

A number of rabbits that received no treatment served as controls. Biopsies of the spleen were performed on several of these, and all animals in this group were killed and necropsied along with the group that was treated with ribonucleate.

As a routine procedure, duplicate blocks were taken post mortem from the heart, lungs, liver, spleen, kidneys, adrenal glands, inguinal and axillary lymph nodes, and sternal marrow. They were fixed in Zenker's fluid and in 10 per cent formalin. In a number of the animals additional blocks were obtained from the tongue, bladder, intestine, and genital organs. The specimens were embedded in paraffin and cut at 4 to 5 μ. Sections were stained routinely with hematoxylin and eosin. The Congo red, cresyl violet, and periodic acid-Schiff staining procedures were employed in order to study the amyloid deposits, and sections of spleen and lymph nodes frequently were stained with eosin and methylene blue in order to facilitate the study of cellular detail in these tissues.

The Development of Amyloidosis

In an initial experiment, 5 rabbits were treated with ribonucleate for 123 days, 10 cc. of a 5 per cent solution being given subcutaneously five times a week. At the end of this period each animal was killed by injecting air into an ear vein, and then subjected to detailed postmortem examination. The severity of amyloidosis of spleen, kidneys, lymph nodes, and liver, graded from 1 to 4 plus on the basis of microscopic study, is shown in Table I.

The spleens were moderately enlarged and firmer than normal. Their cut surfaces were dark red, rather dry, and relatively homogeneous to the naked eye. In two of the five spleens a positive iodine-sulfuric acid test was obtained. Microscopic examination disclosed extensive deposition of amyloid in all five spleens, particularly within the sinusoids about the lymph follicles. Often part or all of the follicles had been replaced by amyloid. In 2 of the 5 animals the process was quite diffuse throughout the spleen. The deposited material generally filled the sinusoids as eosinophilic ribbons or flakes. It proved readily and selectively stainable with Congo red and was metachromatic when stained with cresyl violet, but it appeared to be less homogeneous in

microscopic structure than the amyloid that is commonly seen in man. For example, after lowering the sub-stage condenser of the microscope, it was possible to see that many deposits had a laminated structure. Application of the periodic acid-Schiff routine produced only a pale pink color in the deposits. In the sinusoids the amyloid was generally in close contact with the reticulo-endothelial cells, which were increased in number. In the spleens with the most extensive involvement the lymph follicles were much reduced in size by the

TABLE I

Distribution of Amyloid in Rabbits Treated with Ribonucleate for 123 Days

Rabbit	Amount of amyloid				
	Spleen	Kidneys	Liver	Lymph nodes	Adrenal gland, heart, and duodenum
1	+++	+	o	o	o
2	++++	++	+	+	o
3	++++	++	+	o	o
4	++++	+	o	+	o
5	+++	+	o	o	o

+ = Minimal deposition of amyloid, ++ = moderate deposition, +++ = extensive deposition, ++++ = very extensive deposition of amyloid.

encroachment of amyloid deposits at their periphery, and by the presence of small flakes of amyloid in their centers.

The kidneys of 3 of these animals showed numerous tiny, depressed areas in the cortical surfaces as well as occasional larger ones. Extensive scarring, such as will be described in the following section, was not encountered. Microscopically, deposits similar to those in the spleen were found in scattered glomeruli of all 5 animals. Portions of glomerular tufts were replaced or displaced by these deposits, which had a weaker affinity for Congo red than in the spleen, but were stained equally well with cresyl violet. This material was only faintly positive when the periodic acid-Schiff routine was applied, behaving in this respect also like the splenic deposits. In some instances it appeared to be between glomerular capillary loops as judged from sections that were stained by the periodic acid-Schiff-colloidal iron method of Rinehart and Abul-Haj.[9] For the most part, however, the affected portions of glomerular tufts had been disrupted too severely to allow elucidation of the precise relationship of the deposited amyloid to the glomerular capillary. Often one or two cells with round or oval vesicular nuclei and indistinct cytoplasmic borders could be seen within glomerular deposits. The cytoplasm of these cells stained

weakly with Congo red and cresyl violet. It seemed possible that these cells were swollen endothelial cells, although they also resembled macrophages. In addition to the glomerular lesions, casts often were present in various segments of the renal tubules. These casts did not take the Congo red or cresyl violet stains but were stained deep red with the periodic acid-Schiff routine. There were scattered areas of interstitial fibrosis, most of which were located around the tubules near severely distorted glomeruli. The tubules in these areas commonly were undergoing atrophic changes.

Deposits in liver and lymph nodes were inconspicuous or absent. In the liver they were found between the sinusoidal reticulo-endothelium and the liver cords, while in the lymph nodes of groin or axilla they appeared in close proximity to the sinusoidal reticulo-endothelium. The other organs were not remarkable.

TABLE II

Comparison of Biopsy and Necropsy Findings in Rabbits Treated with Ribonucleate

		Biopsy of spleen			Necropsy				
						Amount of amyloid			
Rabbit	Terminal day of treatment	Day	Amount of amyloid	Giant cells	Day	Spleen	Kidneys	Liver	Lymph nodes
6	136	—	—	—	136	++++	++	+	+
7	104	—	—	—	104	+++	+	o	o
8	176	190	++++	++++	223	+	++++	o	o
9	175	189	+++	+++	223	o	++++	o	o
10	180	200	++++	o	225	++	+	o	o
11	180	202	+++	o	230	o	++	o	o
12	180	200	+++	+++	230	o	++	o	o
13	180	202	++++	o	235	o	++	o	o
14	180	201	++++	o	237	++	+++	o	o
15	180	201	++++	o	237	+	+++	o	o

+ to ++++ = minimal to very extensive deposits.

THE RESORPTION OF AMYLOID

In a second experiment, 10 rabbits were treated with ribonucleate as before. Two of the animals were sacrificed after 104 and 136 days of treatment, respectively. Both proved to have advanced amyloidosis of the spleen and mild involvement of the kidneys (Table II). Their lesions were in every way similar to those described in the previous section.

The remaining 8 animals were treated with ribonucleate during a period of approximately 6 months (Table II). Two to 3 weeks fol-

lowing cessation of treatment, tissue was taken for biopsy from the spleens of these animals. As a control, specimens were taken from the spleens of 4 healthy, untreated rabbits. After a subsequent period of 2 to 5 weeks (Table II) during which no treatment was given, all 12 rabbits were killed and necropsied.

At the time of biopsy the spleens of the 8 treated rabbits were all enlarged, in the majority of cases to about twice the normal size. Several were somewhat firmer than normal. In three instances the splenic biopsies revealed features that had not developed in the previous experiment. In areas that were extensively involved by amyloidosis there were many multinucleated giant cells. Most of these contained eosinophilic material that was indistinguishable from the extracellular deposits of amyloid (Figs. 1, 2, and 3). Much of this intracellular material was readily stainable with Congo red and reacted metachromatically with cresyl violet. Most of it had the somewhat laminar structure of the extracellular amyloid. However, not all of this intracellular material reacted positively to Congo red or cresyl violet nor did it always stain well with eosin. Here and there within giant cells it was fragmented or had a vacuolated, moth-eaten appearance as if parts of it had disappeared, while elsewhere in the same cells its structure and staining characteristics were preserved. Some of the giant cells were huge, measuring 80 to 100 μ in cross section. The majority looked like foreign body giant cells and their nuclei were irregularly distributed within the cytoplasm (Figs. 1, 2, and 3). These histologic details indicated that the giant cells had engulfed amyloid deposits. Their phagocytic activity was further evidenced by the fact that within some of them other structures such as red blood cells, splenic reticulum, and polymorphonuclear leukocytes could be seen with the amyloid (Fig. 4).

The way in which the giant cells were formed was discernible. Here and there, a rim of flat elongated nuclei delineated in a half or three quarter circle a portion of the splenic pulp, together with its content of amyloid and red and white blood cells (Fig. 4). Elsewhere, fragments of splenic structure and amyloid were thus entirely isolated, appearing now as huge giant cells with innumerable, peripherally placed nuclei (Fig. 2). There were also giant cells in which the nuclei were closely crowded together, but in which the cytoplasmic inclusions were relatively homogeneous. It was impossible to be certain whether there was amyloid within the individual mononuclear histiocytes that were loosely scattered within the splenic sinusoids. Within the sinusoids were collections of polymorphonuclear leukocytes, myelocytes

and occasional immature blood cells that could not be identified with certainty. Many plasma cells and an occasional megakaryocyte could be seen. It was noteworthy that only sinusoidal reticulo-endothelial cells, free histiocytes, and giant cells were closely applied to the amyloid. The significance of these findings will be discussed later.

In 5 other rabbits the spleens were much enlarged at operation and much firmer than normal. The biopsy specimens revealed advanced amyloidosis of the type observed in the preceding experiment, without giant cell reaction (Figs. 5 and 7). Thus there was no presumptive evidence of resorptive activity in these five spleens at the time of biopsy. Specimens for biopsy were taken also from 4 untreated control rabbits. Grossly, the spleens of these animals were normal in size and appearance, and they proved to be normal on microscopic examination.

As shown in Table II, the 8 treated animals that had been subjected to a biopsy of the spleen were killed 3 to 5 weeks later. At the time of sacrifice they appeared to be well nourished, and gave no outward signs of illness.

The post-mortem findings in the spleens of these animals will be described first. Care was taken to exclude splenic tissue from the immediate vicinity of the old ligatures. Spleens in which a giant cell reaction had been found previously (rabbits 8, 9, and 12) now were not remarkable grossly. Upon microscopic examination small fragments of amyloid were found in one of these (rabbit 8), while in two others no traces could be discovered (rabbits 9 and 12). In all three there were now many large lymphoid follicles similar to those shown in Figure 6. The centers of the follicles were made up largely of mature lymphocytes (Fig. 8). Situated around each core of lymphocytes was a broad band of monocytes of the type that Maximow has called "polyblasts" (Fig. 10). Scattered among these were polymorphonuclear leukocytes, occasional myelocytes, and a good many plasma cells. In the centers of some of the lymphoid follicles there were a few so-called large reticulum cells. The broad peripheral reactive zones occupied the areas in which amyloid deposition had been most marked at biopsy and in which giant cells had been located previously. Between these perifollicular reactive zones were the dilated sinusoids of the red pulp which often appeared to be almost devoid of cellular content.

The remaining segments of the spleens of the other 5 animals that had been given ribonucleate (nos. 10, 11, 13, 14, and 15) were now of normal size and much softer than at biopsy. It will be recalled that

the specimens taken for biopsy from these rabbits had shown advanced amyloidosis without giant or other reactive cells. Comparisons between the sections for biopsy and the post-mortem sections of these spleens disclosed changes ranging from complete disappearance (as shown in Figs. 5 and 6) to conspicuous diminution of amyloid. Again there were large lymph follicles surrounded by broad bands of large mononuclear cells (Figs. 6 and 10). In two of the five spleens the sinusoidal reticulo-endothelium appeared to have undergone a marked proliferation since the time of biopsy, and in these instances amyloid was still present in considerable quantity. However, there were also scattered foci of extramedullary hematopoiesis (Fig. 11) and of plasma cells. The amyloid was only weakly colored by Congo red, and only in some areas, although in control sections amyloid was turned a brilliant red. Of the remaining 3 rabbits in this group one had a few scattered, broken-up deposits in the spleen (Fig. 12), while in the other two no trace of amyloid could be found. The general microscopic appearance of these three spleens was similar to that of the other spleens in which resorption was complete.

The kidneys of these 8 rabbits were altered extensively. They were paler and somewhat smaller than normal, and the capsules were adherent to the cortices. In several instances the cortical surfaces were finely and diffusely granular (Fig. 9), while in others there were scattered, flat, depressed scars. On section the cortical surfaces were narrowed and contained fine, radial, gray scars. The medullae and pelvices were not remarkable grossly.

Microscopically, the changes were similar to those already described in the preceding section, though in the diffusely contracted kidneys they were of much greater severity and much more generally distributed. In these kidneys only a small number of unaffected glomeruli could be found. There were extensive deposits of amyloid in most of the glomeruli, and in some instances the latter were replaced, partly or wholly, by fibrous tissue (Figs. 13 and 14). It is of importance to note that there was severe renal involvement in several rabbits whose spleens had been freed or nearly freed from amyloid deposits (Table II). In the kidneys of 2 rabbits (nos. 9 and 11, Table II) there were scattered, strikingly enlarged Bowman's spaces, lined by capsular epithelium, but without any glomeruli (Fig. 15). A few of these spaces contained small amounts of a granular, eosinophilic material, probably remnants of the disintegrated glomerular tufts (Fig. 15). Thus it appeared that some glomeruli had undergone lysis and had disappeared in some unknown way. In a few instances the process was

not so complete, there being discernible remnants of the glomerular tufts, together with small amounts of amyloid. It is noteworthy also that the arterioles in the renal cortices of these 2 animals had walls that were considerably thickened owing to hypertrophy of the medial musculature (Fig. 15). This was particularly evident on comparison of these vessels with corresponding arterioles of the untreated control animals.

No amyloid was found in other organs in any of these rabbits. However, in a majority of them the axillary and inguinal lymph nodes were enlarged. On microscopic examination most of these nodes proved to have enlarged lymph follicles with reactive centers, while in their sinusoids there were collections of plasma cells, histiocytes, and occasional polymorphonuclear leukocytes. The changes were most conspicuous in lymph nodes of animals from whose spleens amyloid had disappeared completely or almost completely. Plasmacytosis was generally concurrent in the spleen and lymph nodes. The other tissues of these animals showed no noteworthy changes. In particular, the sternal marrow was not remarkable in any of the rabbits.

Post-mortem examination of the untreated control rabbits revealed nothing of significance. Examination of the remainders of their spleens made it clear that apart from a moderate degree of fibrosis in the immediate vicinity of the ligatures, no reaction had taken place as a result of the excision for biopsy.

Discussion

The experiments here recorded make it plain that amyloid was resorbed completely from the spleens of several rabbits, and to a considerable extent from those of several others. The findings confirm the hypothesis that resorption of amyloid is accomplished, at least in part, by means of an intracellular phagocytic process. Before considering the details of these findings a brief review of the pertinent work of others is in order.

In 1923 Kuczynski[4] reported post-mortem findings in mice that had been treated with nutrose to produce amyloidosis, but had been allowed to survive for some weeks following cessation of treatment. He noted certain peculiarities of the amyloid in the spleens of these animals, such as the heterogeneity of staining when the iodine test was applied. It also appeared to him that the amyloid deposits were being vascularized, and he found an abundance of plasma cells but no evidence of phagocytosis of amyloid in these spleens. Deposits in the liver, however, often were engulfed by giant cells. Kuczynski con-

cluded from these findings that extracellular resorption of amyloid was taking place in the spleens and intracellular resorption in the livers of the animals. Morgenstern[5] criticized the methods used by Kuczynski, but his own report did not take the matter any further, for, in spite of his statement that he performed biopsies on the livers of several amyloidotic mice, he did not report comparisons between the findings at biopsy and post mortem. Reactions similar to those noted by Kuczynski and Morgenstern in mice were seen by Migounow at post-mortem examinations of amyloidotic rabbits. Migounow[6] also mentioned the presence of giant cells in the spleen. By contrast, he saw no reaction to amyloid in the renal glomeruli, and concluded that resorption was taking place in the spleen but not in the kidneys. The same conclusion was reported by Dick and Leiter[7] in a summary of similar studies. In man the inclusion of amyloid in giant cells has been seen repeatedly, particularly in so-called amyloid tumors.[10-12] That the presence of these cells is indicative of a resorptive process has, however, been contested.[11,13] The chief objection to studies such as these has been that there is much variation in the severity of amyloidosis in different animals or individuals, even though the general circumstances under which the disease occurs may be similar or identical. It has been said that the presence of giant cells does not by itself prove that amyloid is being or will be removed from an organ, for such cells may be only reactive.[13] Another objection, emphasized by Letterer,[14,15] has been that the incidence of amyloidosis in experimental animals varies greatly and depends on such factors as diet. With the methods that have been used hitherto to produce the disease in rabbits, the incidence has generally been well below 100 per cent.

In the present experiments the severity of the disease varied, but the incidence proved to be 100 per cent. Scattered but extensive phagocytosis of amyloid in the spleen was followed in the same animals by complete disappearance of the substance from the entire organ. Structural and tinctorial changes in the phagocytized amyloid were evident. Moreover, phagocytosis was not observed in any animals that were given injections of ribonucleate up to the time they were sacrificed, although these rabbits developed extensive amyloidosis. Thus, phagocytosis always followed the cessation of treatment, and it may be postulated that it could not occur while a stimulus for the formation of more amyloid was still being provided. Subsequent to the withdrawal of this stimulus, phagocytosis could take place until all of the amyloid in the spleen had been removed. Moreover, the structural pattern of the spleen, which had been distorted by

the deposits of amyloid, was restored to practically normal. It is reasonable to suppose that the large mononuclear cells (histiocytes, "polyblasts"), which engulfed amyloid in groups, did so individually also; yet an extracellular lysis of amyloid also may have taken place. As is well known, amyloid is quite insoluble in its native state. Drastic means, resulting in its denaturation or decomposition, are necessary to render it completely soluble.

In vivo, the lysis of amyloid is probably accomplished through enzymatic processes. Such processes can be both intracellular and extracellular, and hence the assumption that the lysis of amyloid was accomplished in both ways is not unreasonable. The presence in the spleen of many polymorphonuclear leukocytes, myelocytes, plasma cells, and even a few megakaryocytes during and after the process of resorption was probably a reactive phenomenon that was contributory to the resorptive process. In the biopsy specimens in which they were present, these cells were most often located in areas in which there was no amyloid rather than in the immediate vicinity of amyloid. While mature polymorphonuclear leukocytes may have taken part in the phagocytosis of amyloid or of its breakdown products, the myelocytes, megakaryocytes, plasma cells, and unidentifiable immature blood cells that were present also were probably incapable of phagocytizing particulate matter. They may, however, have promoted the extracellular breakdown of amyloid by the release of enzymes.

Why the cellular hyperactivity in the spleens was so heterogeneous is an interesting problem. It may have been the expression of a powerful non-specific stimulation of the splenic reticulo-endothelium in which the full potential of reticulo-endothelial activity was evoked, with a resulting production of various types of cells including some not concerned with the removal of amyloid. Since the bone marrow was not hyperplastic in any of the animals, it seems likely that the cellular reactions in the spleen were caused by local rather than by general factors.

In the light of recent investigations plasmacytosis in the spleen and lymph nodes during the resorptive process may have been a feature of immunity. For example, products such as peptides, liberated as a result of the break-down of amyloid, may have evoked an anamnestic reaction, *i.e.*, a production of antibodies to antigens with which the animals had had previous incidental contact. The occurrence of plasmacytosis in response to antigenic stimulation in rabbits is a well known fact that has given rise to the concept that plasma cells pro-

duce antibodies.[16-18] It is of interest in this connection that detailed studies of the serum proteins of these rabbits have shown a very significant rise in gamma globulin levels after cessation of treatment with ribonucleate,[19] *i.e.*, during the period when resorption was taking place. Since most antibodies in the rabbit are gamma globulins, the possibility that the production of certain antibodies was stimulated during this period is manifest.

While the experiments left no doubt that resorption of amyloid had taken place in the spleens, they failed to provide an answer with regard to the kidneys. The disintegration and disappearance of many glomeruli, as observed post mortem in two rabbits, could be interpreted either as a resorptive phenomenon or simply as necrosis resulting from the shut-down of the glomerular blood supply. It was clear, however, that the deposition of amyloid in the glomeruli led to extensive glomerular fibrosis while at the same time fibrosis was not a significant part of the changes in the spleen. Furthermore, it was shown that extensive deposits of amyloid had disappeared or nearly disappeared from the spleens at a time when amyloid was still present in abundance in the kidneys. For these reasons it may be assumed that the reabsorption of amyloid depends in large measure upon the milieu that surrounds the amyloid.

The hypertrophy of the renal arterioles that was found in the most severely "contracted" kidneys could have been the result of hypertension. In man and in several species of experimental animals prolonged hypertension often results in this change and as a rule hypertension accompanies chronic diffuse glomerular disease.

Summary

In the experimental studies here described, detailed histologic comparisons of biopsy and post-mortem specimens made it plain that extensive deposits of amyloid were resorbed from the spleens of rabbits.

The amyloidosis that was studied resulted regularly from repeated subcutaneous injections of sodium ribonucleate. The disease always involved spleen and kidneys, and occasionally liver and lymph nodes as well. In a series of 8 rabbits, biopsies of the spleen were performed 2 to 3 weeks following cessation of long-continued treatment with ribonucleate. At this time extensive phagocytosis of amyloid by foreign body giant cells of reticulo-endothelial origin was found in the biopsied spleens of 3 rabbits while in 5 others there was no such phagocytic

reaction though amyloid deposition was extensive. Post-mortem examinations of the same 8 rabbits 3 to 5 weeks following biopsy revealed that amyloid had disappeared completely from the spleens of 4 of the animals, and to a marked degree from those of the other 4 rabbits. Collections of large mononuclear cells with scanty cytoplasm now occupied the areas in which amyloid deposits had been most prominent. These cells were accompanied by scattered foci of extramedullary hematopoiesis. An extensive plasmacytosis also was present in the spleens and lymph nodes of these animals. The kidneys showed advanced amyloidosis post mortem, but failed to provide definite evidence of resorption.

We are indebted to Mr. Julius Mesiar for the photomicrographs.

REFERENCES

1. Waldenström, H. On the formation and disappearance of amyloid in man. *Acta chir. Scandinav.*, 1928, 63, 479–530.
2. Grayzel, H. G., and Jacobi, M. Secondary amyloidosis: results of therapy with desiccated whole liver powder. *Ann. Int. Med.*, 1938–39, 12, 39–58.
3. Rosenblatt, M. B. Recovery from generalized amyloidosis secondary to pulmonary tuberculosis. *Arch. Int. Med.*, 1936, 57, 562–565.
4. Kuczynski, M. H. Weitere Beiträge zur Lehre vom Amyloid. III. (Über die Rückbildung des Amyloids.) *Klin. Wchnschr.*, 1923, 2, 2193–2195.
5. Morgenstern, Z. Zur Frage über Amyloidose und Resorption. *Virchows Arch. f. path. Anat.*, 1926, 259, 698–725.
6. Migounow, B. I. Les faits expérimentaux et anatomiques sur la résorption des substances amyloïdes chez l'homme. *Arch. internat. de méd. expér.*, 1938–39, 13, 437–447.
7. Dick, G. F., and Leiter, L. Some factors in the development, localization and reabsorption of experimental amyloidosis in the rabbit. *Am. J. Path.*, 1941, 17, 741–753.
8. Richter, G. W. Experimental hyperglobulinemia. The effect of injected ribonucleotide on serum globulin levels in rabbits undergoing immunization. *J. Exper. Med.*, 1952, 96, 331–346.
9. Rinehart, J. F., and Abul-Haj, S. K. An improved method for histologic demonstration of acid mucopolysaccharides in tissues. *A. M. A. Arch. Path.*, 1951, 52, 189–194.
10. Schmidt, M. B. Referat über Amyloid. *Verhandl. d. deutsch. path. Gesellsch.*, 1904, 7, 2–18.
11. Meyer, O. Über lokales tumorartiges Amyloid in den Lungen. *Frankfurt. Ztschr. f. Path.*, 1911, 8, 304–318.
12. Abrikossoff, A. J. Ueber Amyloidresorption durch Fremdkörperriesenzellen beim Menschen. *Centralbl. f. allg. Path. u. path. Anat.*, 1934–35, 61, 193–195.
13. Fischer, W. Ueber Fremdkörperriesenzellen bei Amyloid der Milz. *Centralbl. f. allg. Path. u. path. Anat.*, 1910, 21, 49–54.
14. Letterer, E. Studien über Art und Entstehung des Amyloids. *Beitr. z. path. Anat. u. z. allg. Path.*, 1926, 75, 486–588.

15. Letterer, E. Neue Untersuchungen über die Entstehung des Amyloids. *Virchows Arch. f. path. Anat.*, 1934, **293**, 34–72.
16. Bjørneboe, M., and Gormsen, H. Experimental studies on the rôle of plasma cells as antibody producers. *Acta path. et microbiol. Scandinav.*, 1943, **20**, 649–692.
17. Bjørneboe, M., Gormsen, H., and Lundquist, F. Further experimental studies on the rôle of the plasma cells as antibody producers. *J. Immunol.*, 1947, **55**, 121–129.
18. Fagraeus, A. Antibody production in relation to the development of plasma cells. *Acta med. Scandinav.*, 1948, suppl. 204, 122 pp.
19. Richter, G. W. To be published.

[*Illustrations follow*]

LEGENDS FOR FIGURES

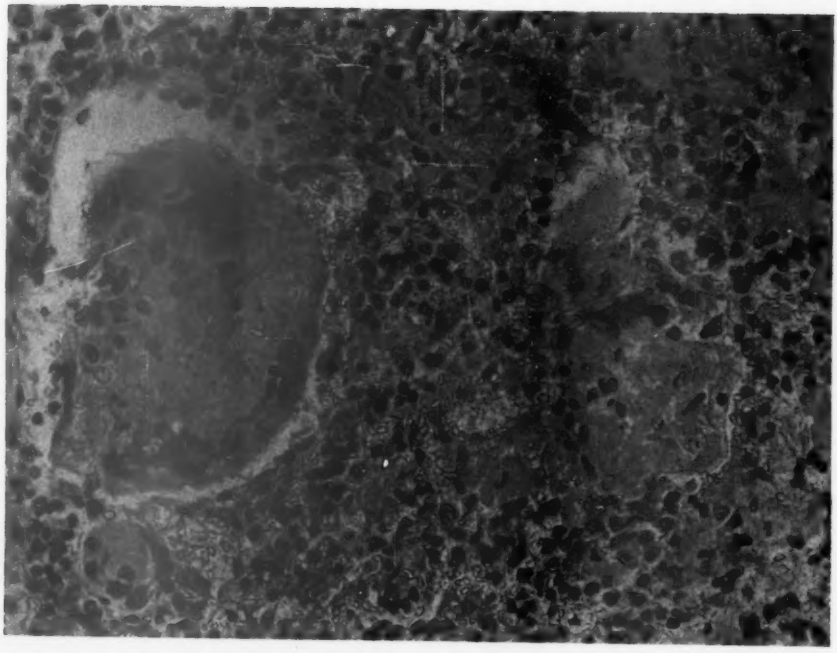
FIG. 1. Biopsy specimen from the spleen of a rabbit (no. 9, Table II) 2 weeks following cessation of treatment with ribonucleate. A huge, multinucleate giant cell containing amyloid is shown at the left. There are extensive deposits of amyloid in the sinusoids. Congo red and hematoxylin stains. × 280.

FIG. 2. From the same specimen as that from which Figure 1 was made. A somewhat different type of giant cell contains laminated amyloid, part of which stained salmon pink with Congo red. Congo red and hematoxylin stains. × 450.

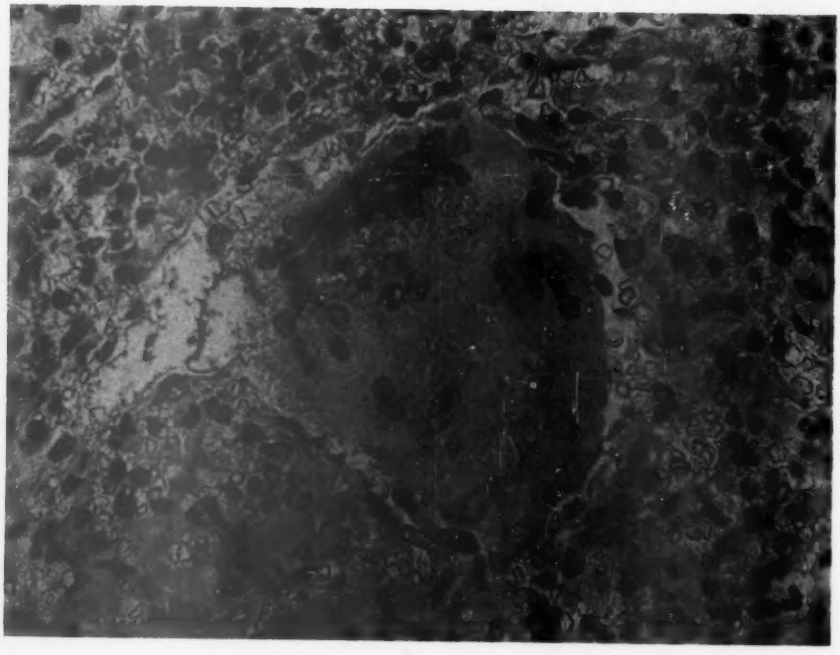
V3(2)
MAR
APR
54
XU

1

2



1

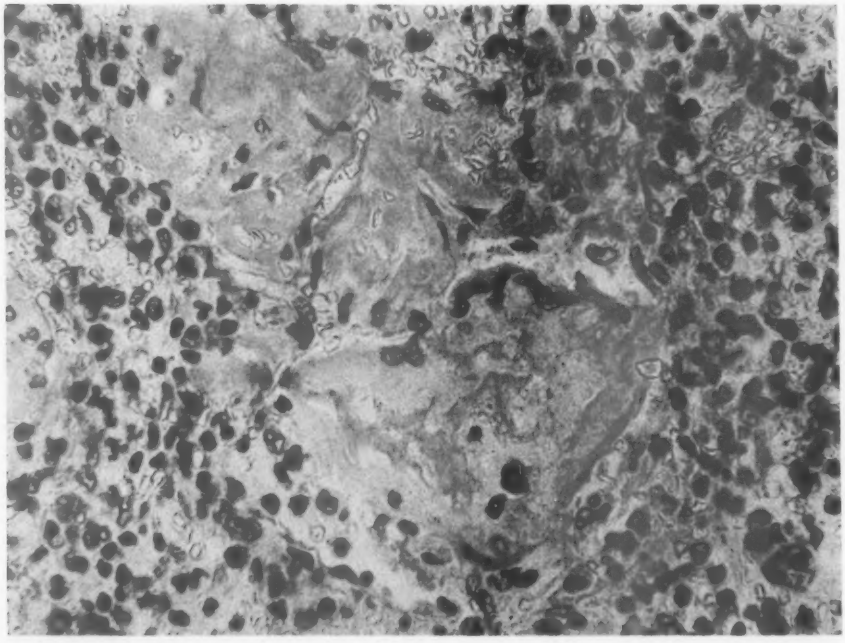


2

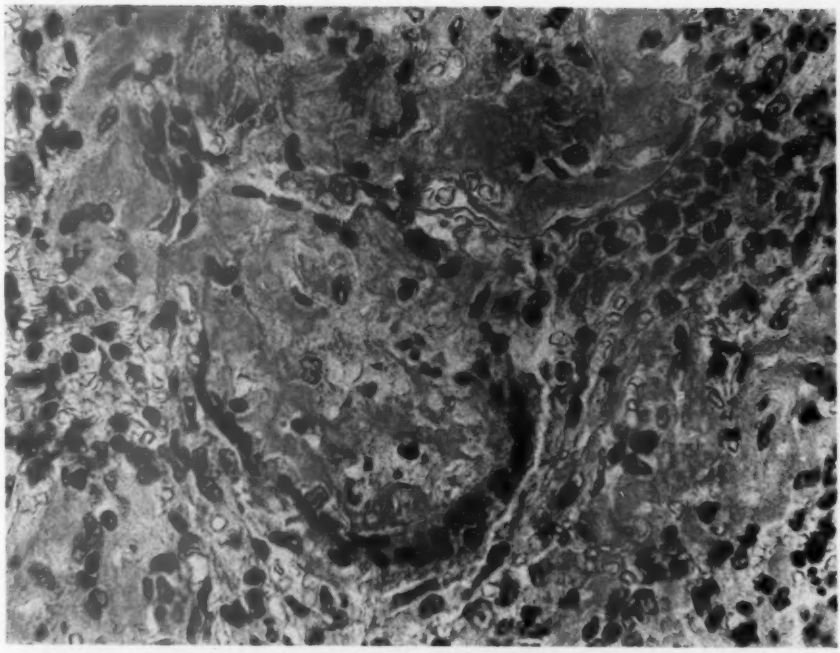
FIG. 3. From the same specimen as that from which Figures 1 and 2 were made. In the lower portion of the field the formation of a giant cell is taking place. Large mononuclear cells (histiocytes?) have applied themselves to a deposit of amyloid. The latter is not stained uniformly and appears to be more heterogeneous than the deposits at the top of the field. Congo red and hematoxylin stains. × 400.

FIG. 4. From the same specimen as Figures 1 to 3. A segment of the splenic pulp containing amyloid, erythrocytes, and fragments of other cells is being isolated by proliferating sinusoidal reticulo-endothelium. Congo red and hematoxylin stains. × 450.

FIG. 5. Biopsy specimen from the spleen of a rabbit (no. 13, Table II) 3 weeks after cessation of treatment with ribonucleate. The red pulp is filled with amyloid, and the lymphoid nodules have been extensively replaced by it. Of note is the absence of giant cells. Hematoxylin and eosin stain. × 50.



4

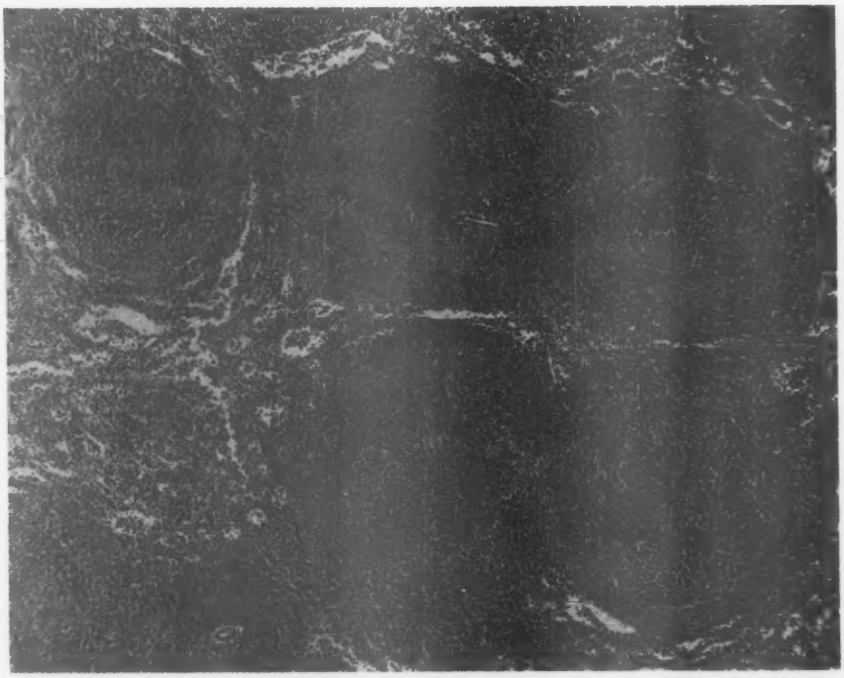


5

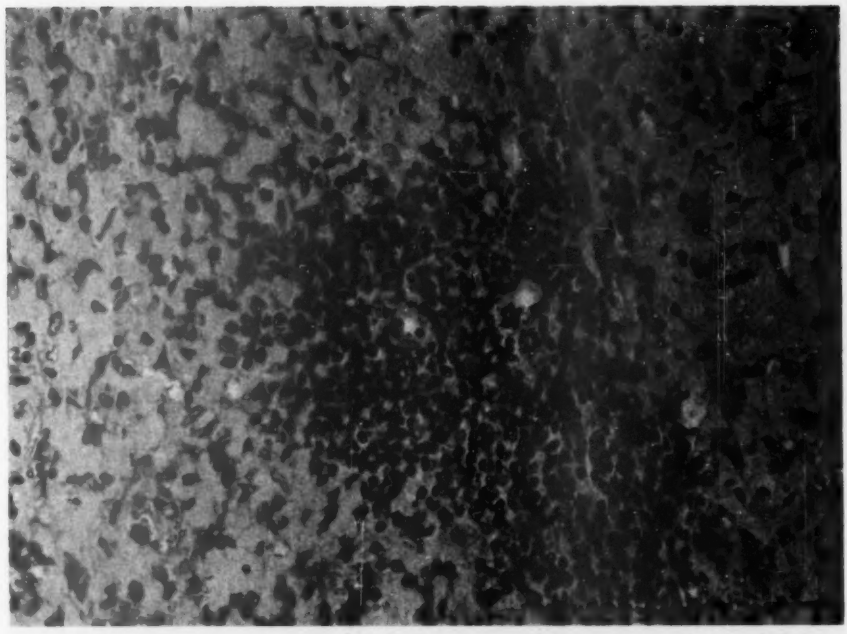
FIG. 6. Spleen of the same rabbit as Figure 5, after death of the animal 32 days later. All amyloid has disappeared. There are now many large lymphoid nodules with outer zones composed of large mononuclear cells. Hematoxylin and eosin stain. × 50.

FIG. 7. Detail of Figure 5. × 280.

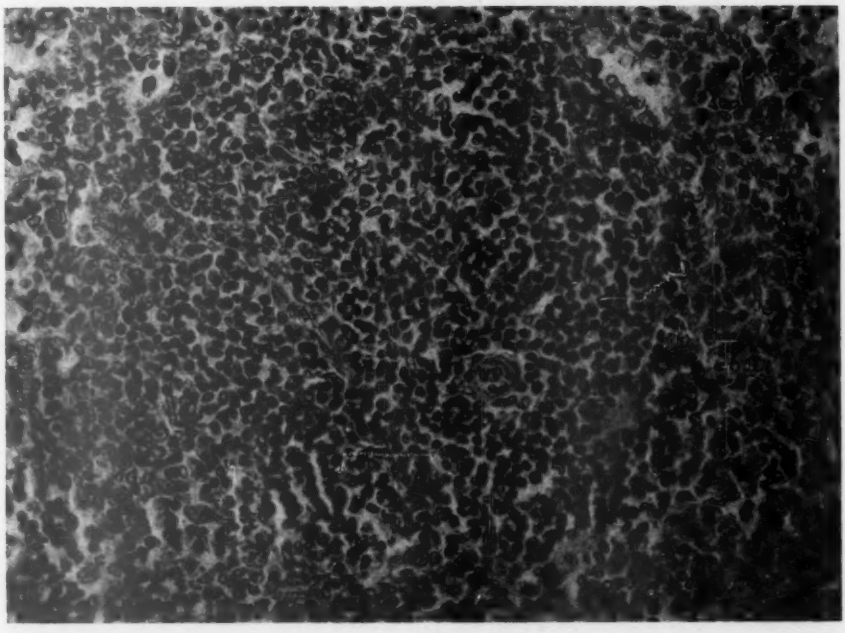
FIG. 8. Detail of Figure 6, for comparison with Figure 7. Complete absence of amyloid may be noted. × 280.



6



7



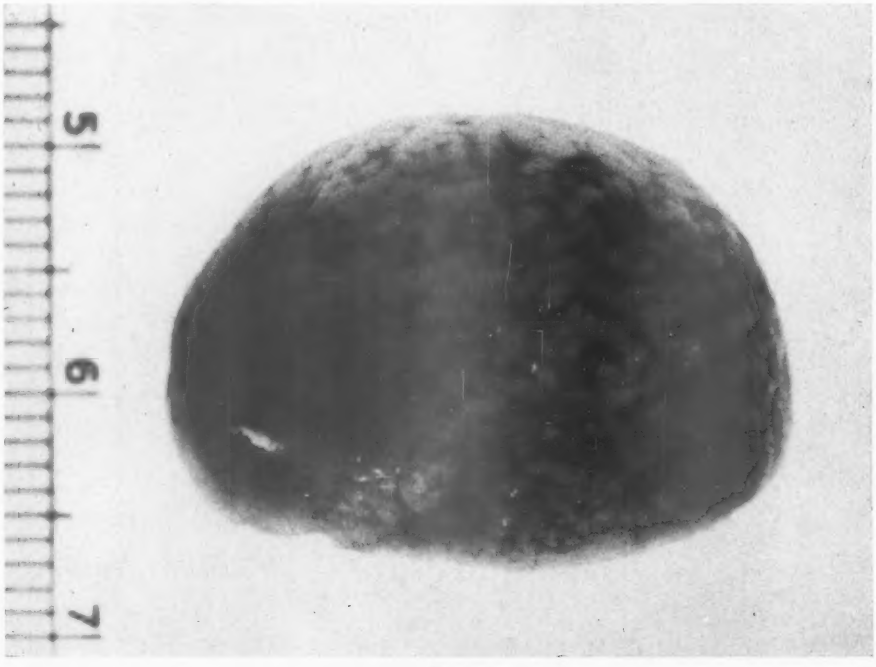
8

FIG. 9. A contracted, amyloidotic rabbit kidney. The cortical surface is diffusely granular. × 3.7.

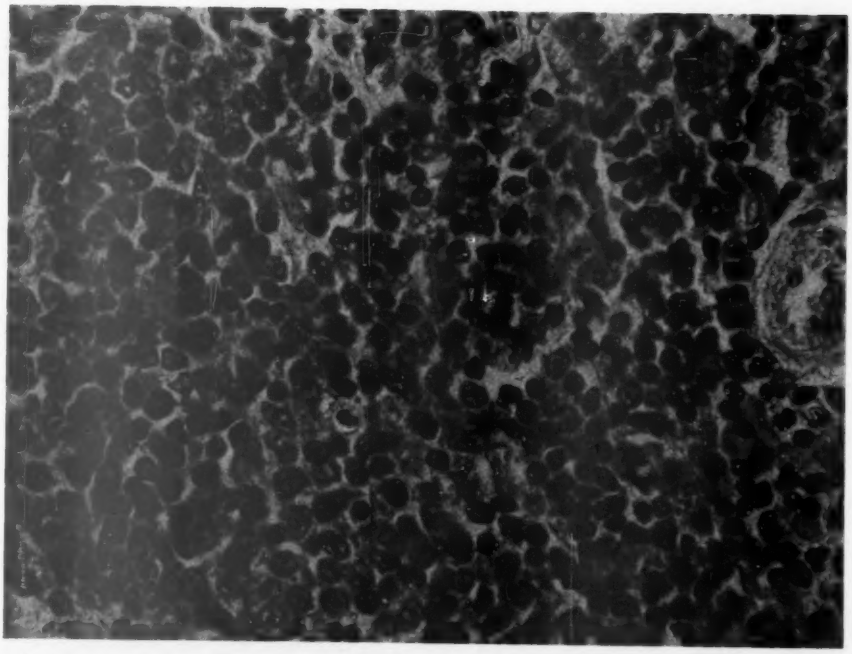
FIG. 10. Another detail of Figure 6 showing the many large mononuclear cells with scant cytoplasm at the periphery of a lymphoid nodule following the resorption of amyloid. × 600.

FIG. 11. Spleen. Necropsy specimen showing extramedullary hematopoiesis. Myelocytes are prominent. Most of the amyloid in this spleen has been resorbed. Eosin and methylene blue stains. × 1130.

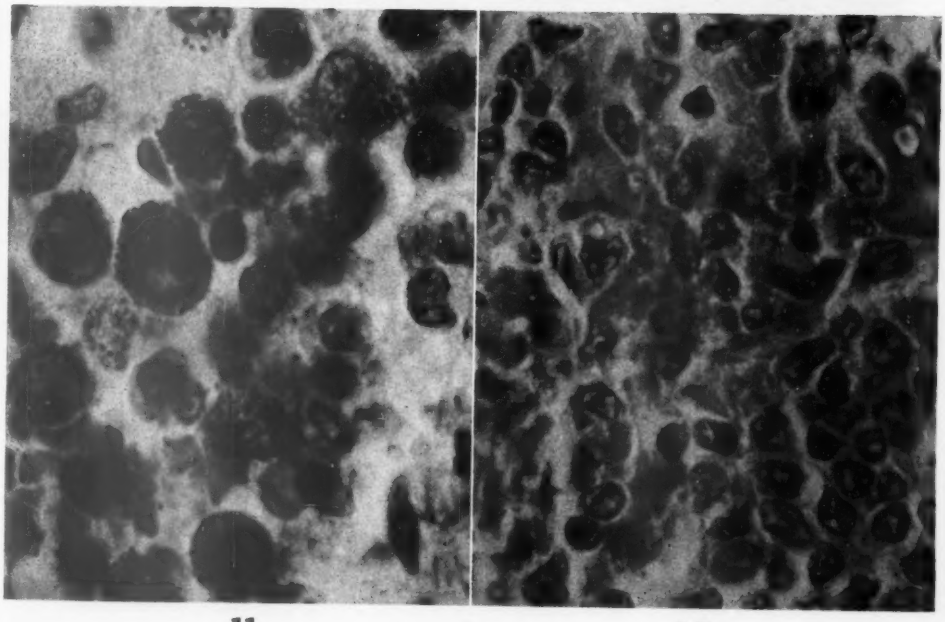
FIG. 12. Necropsy specimen of spleen (rabbit 8, Table II). Resorption of amyloid was incomplete. Fragments of amyloid are scattered between monocytes at the periphery of a lymphoid nodule. Congo red and hematoxylin stains. × 970.



9



10

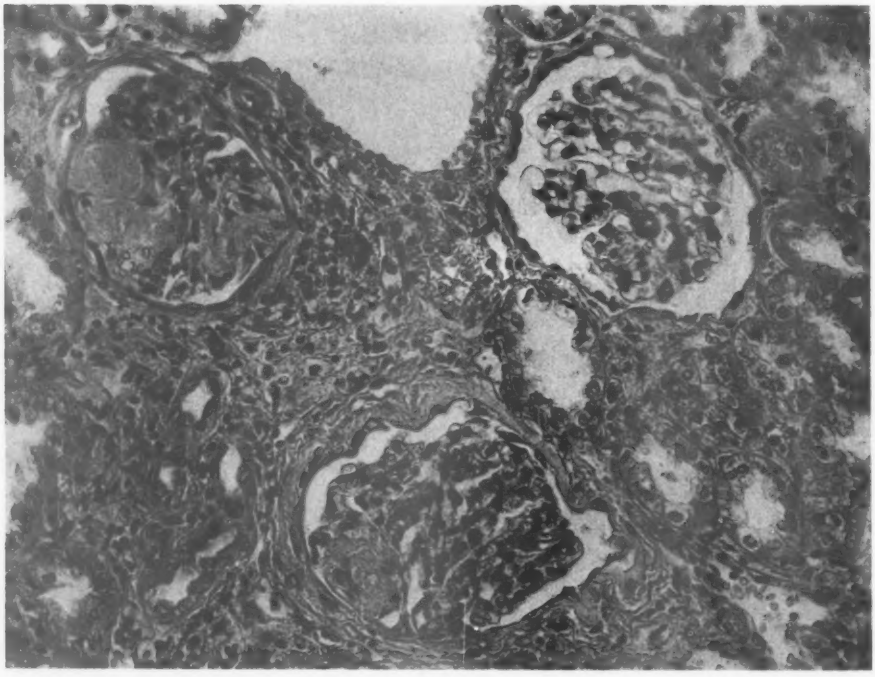


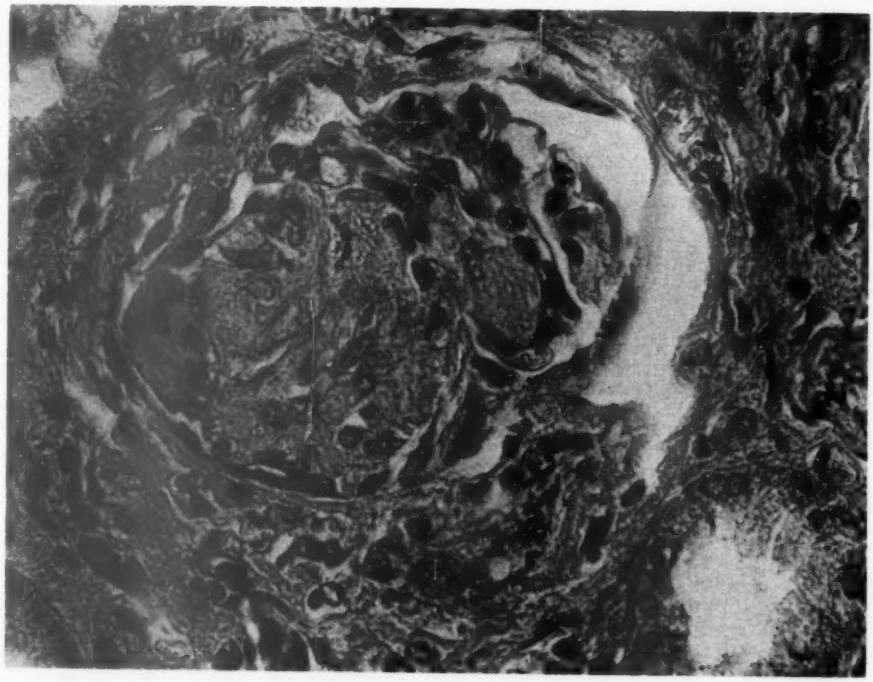
11 12

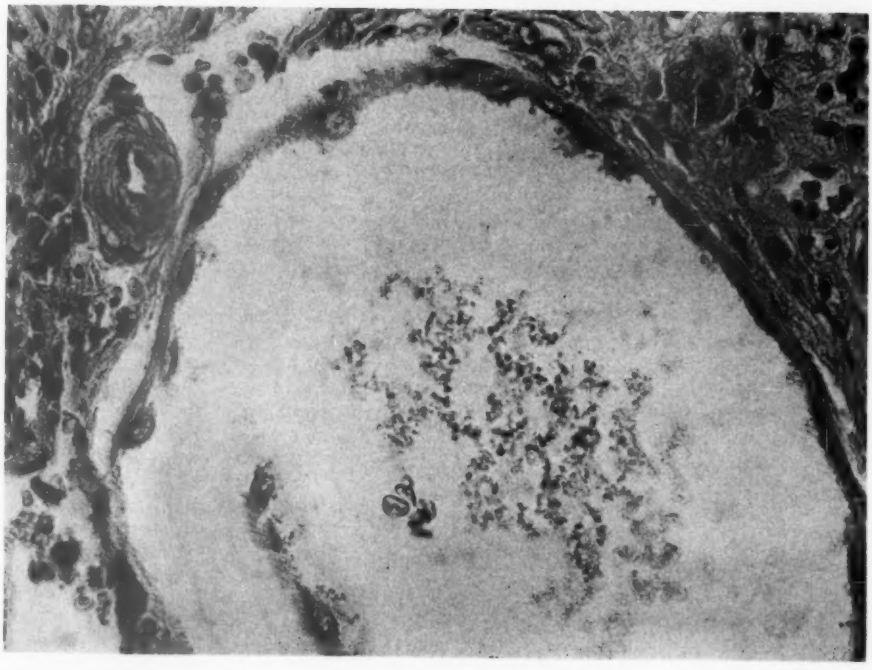
FIG. 13. Section of the kidney shown in Figure 9. There are deposits of amyloid in two of the three glomeruli, and pericapsular fibrosis. No evidence of resorption. Hematoxylin and eosin stain. × 240.

FIG. 14. Rabbit's kidney. A glomerulus has been greatly altered by deposition of amyloid, adhesion of tuft and capsular epithelium, and early fibrosis. In the spleen of this rabbit amyloid had been completely resorbed. Hematoxylin and eosin stain. × 600.

FIG. 15. From the same section as Figure 14. Here a glomerular tuft has disappeared completely, leaving a dilated Bowman's space containing granular material and desquamated capsular epithelial cells. There is marked hypertrophy of the arteriole near the left upper corner of the field. × 600.







ADULT LIPIDOSIS RESEMBLING NIEMANN-PICK'S DISEASE *

ROBERT D. TERRY, M.D., WARREN M. SPERRY, PH.D., and
BERNARD BRODOFF, M.D.

(From the Divisions of Laboratories and of Medicine, Montefiore Hospital, and the New York State Psychiatric Institute, New York, N.Y.)

This report presents a detailed clinical, anatomical, and chemical study of an unusual case of lipidosis. The patient was a 52-year-old male who died 13 months after the onset of an illness, which was characterized by particularly severe involvement of the lungs. Histologic study before and after death suggested that this might be Niemann-Pick's disease in an adult. Chemical analysis of the lipid content of the lung, liver, and spleen indicated that the predominant lipid was sphingomyelin but that it was present in somewhat smaller amounts than previously reported in this condition. The tissue concentrations of certain other lipids were not only significantly higher than normal, but also higher than those previously reported in Niemann-Pick's disease in infants and adults. Accordingly, this case does not appear to fit precisely into any of the usual categories of lipidosis.

REPORT OF CASE

B. G., a 53-year-old white Jewish male, was admitted to Montefiore Hospital on December 7, 1951. His illness had begun 10 months previously with the gradual onset of weakness and easy fatigability. After 4 months the patient developed a persistent, hacking cough, productive of moderate amounts of sputum. There were no accompanying chills, fever, or night sweats. On several occasions he coughed up small amounts of blood. Because of this, he entered Greenpoint Hospital in Brooklyn. The sputum and gastric aspirates were negative for acid-fast bacilli. After 5 weeks, the patient was discharged to a convalescent home. While there, dyspnea and orthopnea first appeared. During the ensuing weeks he had several episodes of severe substernal pain with radiation to the left shoulder. On August 7, 1951, during one of these episodes, the patient was admitted to the Presbyterian Hospital in New York.

At that time, extreme dyspnea, cyanosis, and hepatosplenomegaly were noted. The venous pressure was 220 mm. of water. The patient's cardiac failure was successfully treated by the usual regimen, including mercurial administration and digitalization. Roentgenograms of the chest showed pulmonary fibrosis. Biopsy of a lung confirmed this and revealed collections of intra-alveolar foam cells. Radiotherapy to a small portion of the right lung was begun, and five treatments were given. On December 7, 1951, the patient was transferred to Montefiore Hospital.

Although objective data were not available, the patient's family characterized him as having been a dull, backward, sickly child who did not speak until he was 7 years old. At the age of 44 years, he had pneumonia which cleared completely. He was exposed to ground glass dust for 1 year while employed in a tinsel factory at the

* Received for publication, July 2, 1953.

age of 34 years; and he used oily nose drops during the hay fever season from the age of 29 years on.

His father died of asthma at 50, his mother of heart disease at 39 years, and one brother of carcinoma. Three of four living siblings were examined and presented no physical abnormalities.

Physical examination on admission to Montefiore Hospital revealed a well developed, thin, apprehensive, middle-aged white male. Although cooperative and responsive, the patient appeared to be of below average intelligence. He appeared to be chronically ill but was not acutely distressed. The hair was scanty but normally distributed. The skin was cold and without icterus or xanthomas. There were no skeletal abnormalities. The eyes were prominent but without true exopthalmos, and the lower eyelids were puffy. The ocular fundi revealed only slight arteriolar narrowing. The oropharynx was dusky red and dry, with a thick, tenacious, mucoid discharge on the soft palate. The trachea was deviated to the right. The lungs were clear to percussion but presented scattered inspiratory crackles and wheezes. The point of maximal impulse of the heart was not determined, but the heart sounds were loudest in the fifth intercostal space to the right of the sternum. The cardiac rhythm was regular. The second pulmonic sound was greater than the second aortic sound which was split. The blood pressure was 104/86 mm. of Hg. The liver edge was smooth, firm, and non-tender. It was palpable 6 fingersbreadth below the right costal margin. The tip of the spleen was also 3 fingersbreadth below the costal margin. Bilateral inguinal hernias were noted. There was minimal clubbing of the fingers and a slight cyanotic tinge of the finger tips and toes. There was no edema, and peripheral arterial pulsations were good. The neurologic examination was not remarkable.

Laboratory data were as follows: the hemoglobin was 17 gm. per cent; red blood cell count, 5.9 millions per cmm.; white blood count, 7300 per cmm., with a normal differential; platelet count, 212,000 per cmm.; erythrocyte sedimentation rate, 5 mm. per hour (Cutler method). Bleeding and clotting times were normal. Urinalysis revealed a trace of albumin and occasional white blood cells. Bacteriologic studies on sputum for acid-fast bacilli, other bacterial pathogens, and fungi were negative. Tuberculin tests, the Kveim test, and skin tests for blastomycosis, coccidioidomycosis, and histoplasmosis were all negative. Blood urea nitrogen was 16.6 mg. per cent and the fasting blood sugar was 88 mm. per cent. Total serum lipids were 850 mg. per cent; phospholipids, 202.8 mg. per cent; cholesterol, 241.0 mg. per cent. On a subsequent examination, cholesterol was 224 mg. per cent, 69 per cent of which was esterified. The prothrombin time, cephalin-cholesterol flocculation, and thymol turbidity tests were within the normal range. Sternal marrow aspiration revealed many enlarged cells with foamy sudanophilic cytoplasm. Splenic biopsy demonstrated an extensive infiltration by similar cells.

The electrocardiogram revealed digitalis effects and prominent Q waves in leads I, II, AV_L, V-5, and V-6. These were interpreted as evidence of a probable old anterior wall infarction. Voltage was normal. Roentgenograms of the chest demonstrated an extensive reticulated pattern symmetrically covering the lower two thirds of both lung fields. The trachea was displaced to the right as was the cardiac silhouette, its greater portion lying in the right hemithorax. A bone survey was negative.

The patient's course in the hospital was characterized by chronic cough productive of moderate amounts of tenacious sputum and by complaint of extreme dryness of the mouth and oropharynx. The patient was maintained on a low salt diet and digoxin. Shortly after admission the venous pressure was 150 mm. of water and the decholin circulation time was 13 seconds. On March 18, the patient complained of severe left-sided chest pain, worse on respiration, and the sputum was blood-streaked. Severe dyspnea and cyanosis developed. An icteric tinge of the conjunctivae was noted and serum bilirubin determination at that time showed 3.3

mg. per cent. Serum electrolyte values indicated mild respiratory acidosis. The patient was started on intermittent oxygen therapy and antibiotics, receiving penicillin, chloromycetin, and streptomycin in various combinations until death. The dyspnea lessened following mercurial medication. Ankle edema and distended neck veins did not appear. The patient remained cyanotic, became stuporous, and finally lapsed into coma. On the final day of illness, the temperature, which had been normal, rose to 106° F. and Cheyne-Stokes respiration developed. It was believed that the cause of death was severe, prolonged, pulmonary insufficiency.

POST-MORTEM EXAMINATION

Gross Findings

Necropsy was performed 1½ hours post mortem. The body was that of a small, poorly nourished, white male measuring 60 inches in length and weighing an estimated 100 lbs. A yellow tinge was noted over the malar prominences. There were no cutaneous lesions. There was moderate cyanosis and clubbing of the finger tips. The liver edge was palpable 4 fingersbreadth below the right costal margin. The spleen was not palpable.

Lungs. The right lung weighed 950 gm. and the left, 800 gm. Both failed to collapse on a flat surface and were firm except at the apices where there was moderate emphysema. The pleural surfaces had a green-gray color and were spotted with minute, yellow points. The cut surfaces were green-brown with a light speckling of anthracotic pigment. All lobes were subcrepitant. Easily expressible from the cut surfaces was a frothy, greasy material which did not float on water. The trachea and bronchial tree were normal, while the pulmonary arteries showed minimal atherosclerosis. Hilar and tracheobronchial lymph nodes were moderately enlarged and succulent.

Heart. The heart weighed 280 gm. It was of normal shape and symmetry except for mild hypertrophy and dilatation of the right ventricle. The endocardium was smooth and translucent. The valve cusps and orifices were normal. A large, silvery, myocardial scar was found in the posterolateral aspect of the left ventricle. Elsewhere, the myocardium was homogeneously dark red and of normal consistency. The left coronary artery, which was predominant, was noted to be obstructed by an old arteriosclerotic plaque near its origin and again in its circumflex branch. The walls of the aorta and other major arteries were arteriosclerotic, while the major veins were normal.

Liver. The liver weighed 1700 gm. and was of normal shape and consistency, with sharp edges and a smooth surface. The architectural markings of the cut surfaces were lost in a diffuse yellowish brown color. There was no evidence of fibrosis. The intrahepatic and extrahepatic bile ducts and gallbladder were normal.

Spleen. The spleen was enlarged to 600 gm., measuring 16 by 10 by 7 cm. It was of normal shape, purple, and of firm consistency. Cut surfaces presented a beefy red appearance, completely without the usual structural features. A sharply demarcated sphere of darker colored but otherwise similar splenic tissue, 5 cm. in diameter, was found deep within the parenchyma.

Adrenal Glands. The right adrenal gland weighed 11 gm. and the left, 26 gm. Cut sections of the enlarged but normally shaped glands revealed markedly thickened and irregularly outlined opaque, yellow cortices. The medullary tissue was approximately normal in amount and appearance.

Genito-urinary System. The right kidney weighed 110 gm. and the left, 130 gm. Their surfaces were finely granular and were marked by numerous small retention cysts. Cut sections failed to demonstrate any abnormalities. The bladder also was normal, while the prostate was moderately enlarged and nodular. There was a hydrocele on the left and the corresponding testicle was fibrotic, while the right testis was normal.

Bone. Specimens of calvarium, ribs, sternum, and lumbodorsal vertebrae showed normal bony structure and marrow.

Brain. The brain weighed 1040 gm. and was not remarkable grossly except for minimal cerebral edema. The pituitary gland weighed 400 mg. and was of normal appearance.

The thyroid and parathyroid glands, larynx, pancreas, and gastrointestinal system were normal.

Microscopic Examination

Lungs (Figs. 1 to 3). Great numbers of lipid-containing histiocytes were found in the pulmonary alveoli and to a lesser extent in the septa, which were strikingly sclerotic. The intra-alveolar cells were large (30 to 60 μ), polyhedral or oval, with sharply defined cytoplasmic membranes. In paraffin sections stained with hematoxylin and eosin, the cytoplasm was occupied by an acidophilic foam which spread diffusely throughout the cell. This foamy appearance was best seen in preparations stained with Mallory's aniline blue, in which the foam took on a gray-blue color. Frozen sections treated with Nile blue sulfate revealed blue-purple cytoplasmic granules. Smith-Dietrich preparations showed small amounts of phospholipid in these cells. The nuclei were quite large, with a diameter almost one fourth that of the cell. There were commonly two or three, but never more than four nuclei within a single cytoplasmic membrane. The chromatin was

moderately heavy and occasionally a small nucleolus was present. Nuclear outlines were generally ovoid but bizarre shapes were observed also. Mitotic figures among the histiocytes were very rare.

The lipid-containing cells in the septa were somewhat different from those in the alveoli. They were slightly smaller, with less cytoplasm and less distinct outlines. Those along the bronchi and larger vessels often contained yellow, granular pigment. The cytoplasm of these septal histiocytes became brick red when treated with Sudan IV and took on a deep blue color with the Lorrain Smith (Nile blue sulfate) reaction, contrasting with the purple of the intra-alveolar cells. High concentrations of phospholipid were demonstrated in the septal cells by strongly positive Smith-Dietrich preparations.

The septa of those alveoli filled by fat-containing cells were often lined by a closely adherent row of regular, low cuboidal, epithelial cells with eosinophilic cytoplasm and hyperchromatic nuclei. Many areas showed focal pneumonia with acute purulent exudate in small groups of alveoli. The bronchi were often filled by a similar exudate. The small pulmonary arteries and arterioles demonstrated slight hyperplasia of the subintimal layer.

The several special stains used to elucidate the nature of the lipid are presented in Table I. The yellow pigment, although insoluble in

TABLE I

Response of Histiocytic Cytoplasm to Various Staining Techniques

Stain	Histiocytic cytoplasm
Hematoxylin and eosin (paraffin)	pale purple bubbles
Mallory's aniline blue (paraffin)	fine gray-blue bubbles
Sudan IV (frozen)	orange-brown granules
Nile blue (frozen)	deep blue or blue-purple granules
Smith-Dietrich (frozen)	fine blue-black granules

xylene and alcohol, was stained by Sudan IV and Nile blue sulfate. Its reactions with certain other dyes are listed in Table II.

Liver (Fig. 4). In most sections of the liver there were numerous aggregates of swollen, lipid-filled histiocytes. These cells filled the sinusoids, compressing the liver cord cells. The cytoplasm of the parenchymal cells was often ballooned and vacuolated. In hematoxylin and eosin sections these parenchymal and Kupffer cells were thus almost indistinguishable except for their nuclei. Nile blue sulfate produced a deep blue color in the histiocytic cytoplasm, while small, dark blue granules were seen in the cord cell cytoplasm. The lipid proved

to be strongly basophilic in frozen sections. Smith-Dietrich preparations showed that the Kupffer cells held high concentrations of phospholipid, while the parenchymal cells contained very little if any. Intracellular pigment, similar to that seen elsewhere, also was common in the liver. In a few lobules there were foci of relatively recent necrosis with aggregates of polymorphonuclear leukocytes surrounded by congested sinusoids. These foci had no particular relationship to the central veins. There was no evidence of periportal fibrosis nor was the capsule thickened.

TABLE II

Variation in Response of Insoluble Cytoplasmic Pigment and Soluble Lipid to Various Staining Techniques

Stain	Insoluble cytoplasmic pigment	Soluble lipid
Acid fast methods		
Kinyoun	deep blue vague granules	pale blue bubbles
Ziehl-Neelsen	deep blue vague granules	pale blue bubbles
Schiff's periodic acid	deep red-purple granules	pale purple bubbles
Pappenheim pyronine green	deep blue granules	pale blue bubbles
Hemofuscin	dark red granules	bright purple bubbles
Ferrocyanide (Gomori's iron)	negative	negative

Spleen (Fig. 5). The normal splenic components were almost entirely replaced by small groups of large histiocytes similar in appearance to those in the pulmonary alveoli. Smith-Dietrich preparations demonstrated large concentrations of phospholipids. Occasionally small lymphoid follicles persisted around trabeculae or near the larger blood vessels, but for the greater part only narrow columns of normal elements remained to separate the clumps of fat-containing cells. The large, discrete nodule, described grossly, was found to be composed of tissue not much different from that surrounding it, but with even fewer of the normal white pulp elements and with extensive hemorrhage. Although the border between the two areas was quite sharp, there was no capsule. Intracellular pigment was very rare in the spleen.

Genito-urinary System. There was mild arterial nephrosclerosis with focal hyalinization of glomeruli and tubular atrophy. A single, small calcific deposit was found in the cortex of the right kidney. Tubular epithelial cells frequently contained large amounts of yellow-brown granules. The nuclei of these cells were usually normal, although in cells with particularly heavy deposits of pigment they were sometimes hyperchromatic and shrunken. The bladder was not remarkable

while the prostate showed minimal, benign, nodular overgrowth. Normal spermatogenesis was seen in the right testis, but the left was fibrotic and atrophied. An unusual amount of pigment was found in and around the interstitial cells. This pigment could not be differentiated from that normally present here but did appear to be increased.

Lymph Nodes (Fig. 6). All nodes examined were of normal structure except for the frequent occurrence of lipid-containing and pigment-containing cells arranged singly and in groups. These were found with equal frequency in the follicles and in the reticular tissue.

Pituitary Gland. The anterior lobe of the hypophysis revealed intact cells with a normal distribution. The posterior lobe was architecturally unremarkable, but there was an unusual amount of yellow-brown pigment in and around the pituicytes. As in the testes, there seemed to be an increased amount of pigment, but it could not be differentiated qualitatively from that occurring normally. The pigment here stained as elsewhere except for a negative Schiff reaction.

Adrenal Glands (Fig. 7). The adrenal cortices were greatly widened and nodular. Most of the cortical cells were moderately ballooned with an excessive amount of pale, finely vacuolated, and granular cytoplasm. The nuclei were normal. There were also many sharply defined groups and streaks of much enlarged, polyhedral cells measuring 40 to 80 μ in greatest diameter. The cytoplasmic membranes were often ruptured in these foci. These cells frequently failed to present nuclei, but when visible they were indistinguishable from those of the relatively normal cells. In other areas there were groups of moderately enlarged cells with heavy cytoplasmic deposits of yellow pigment. Smith-Dietrich preparations demonstrated the presence of phospholipids in many groups of cells. The cytoplasm of the moderately ballooned cells stained deep blue with Nile blue sulfate while that of the largest cells appeared to have a slight red tinge. Sections of a normal fatty adrenal gland stained as a control showed the presence of more triglycerides as indicated by prominent, bright red globules. Medullary tissue was normal.

Bone (Fig. 8). Multiple sections of ribs, sternum, and vertebrae revealed normal bony trabeculae and cortices. All hematopoietic marrow elements were hyperplastic. Scattered about singly and in groups were swollen, lipid-containing histiocytes with a great deal of cytoplasmic pigment.

Heart. Sections of the posterolateral portion of the left ventricular wall showed an old, dense scar. Elsewhere throughout the myocardium were multiple small, irregular foci composed of a pale, eosinophilic

syncytium of amorphous and fibrillar material without striations or nuclei. These areas had no particular relationship to the myocardial blood vessels. The amorphous material had the staining reactions of amyloid when tested with Congo red and methyl violet. Many of the remaining myocardial cells showed the common paranuclear lipochrome pigment. The larger coronary arteries were marked by patchy atherosclerosis and calcification. The aorta was normal except for minimal thickening of the vasa vasorum.

Brain. There was moderately heavy deposition of sudanophilic material in the neurons of the cerebral cortex, lateral geniculate body, basal ganglia, and Purkinje and granular cell layers of the cerebellum. Similar material was found in association with cortical glia. The fatty substance was found in granules often clumped on one side of the nucleus, but occasionally surrounding it. The cells were minimally ballooned and the nuclei were pushed to an eccentric position by the lipid. Similar sudanophilic granules were noted in vascular endothelial cells. The lipid material did not have the pink color said to be characteristic of gangliosides in Bial preparations,[1,1a] nor did it stain with the Smith-Dietrich reaction. There was a slight loss of cells from the Purkinje layer but gliosis was not found here or in any of the other areas.

Miscellaneous. Within the interstitial tissue of the thyroid gland were occasional cells containing yellow pigment. Sections of pancreas, stomach, psoas muscle, and abdominal skin were normal. Amyloid was not found in any tissue other than the heart.

Comment

In summary, there was massive involvement of the lungs, liver, and spleen by lipid-filled histiocytes, while relatively fewer numbers of these cells were found in the adrenal cortices, bone marrow, and lymph nodes. Abnormal pigment was found in these organs, the thyroid gland, and renal epithelium. Pigment otherwise indistinguishable from the normal was present in excessive amounts in the posterior pituitary lobe and interstitial cells of the testes. The brain demonstrated changes consistent with severe, prolonged anoxia or with any chronic disease. It was evident from the tissue changes that this was a bizarre case of systemic lipidosis.

Christian-Schüller disease involves cholesterol metabolism and is characterized by the succession of proliferative, granulomatous, xanthomatous, and fibrous stages. As this case presented none of these features, this diagnosis could be definitely excluded.

The clinical course and histologic findings, while atypical, suggested

either Gaucher's or Niemann-Pick's disease. In the adult form of Gaucher's disease, the excess cerebroside is localized in the spleen, liver, lymph nodes, and bone marrow. Pulmonary involvement has been reported in only three adult cases.[2,2a] Niemann-Pick's disease is caused by abnormal metabolism and storage of the diamino-phospholipid sphingomyelin. It is almost invariably fatal before the age of 2 years. These infants show a generalized organ involvement including the lungs and tissues of epithelial origin. There have been two chemically authenticated cases in adults. These were brothers reported by Dusendschon[3] and Pfändler.[4] The distribution of lipids in our case is consistent with either Gaucher's or Niemann-Pick's disease. There was, however, great histologic similarity between this case and the two adults mentioned. The only notable anatomical difference was the presence of cirrhosis in those patients. It was a so-called annular cirrhosis with irregular fibrous bands extending from the periportal areas, which contained abnormally numerous biliary tubules. It is to be noted that both brothers had a history of alcoholism, which was not the case with our patient.

The cytologic and histochemical findings are of more value in differentiating between Gaucher's and Niemann-Pick's diseases. The finely vacuolated cytoplasm of the histiocytes is reminiscent of the latter. The Gaucher cell usually has a reticulated or wrinkled appearance. Furthermore, Gaucher cells often have five to eight nuclei while no more than three were found in this case. The results of the Smith-Dietrich and the Mallory aniline blue stains also resembled those observed in Niemann-Pick's disease in infants. The Nile blue sulfate and Smith-Dietrich preparations indicated a predominance of phospholipids in the pulmonary septa, spleen, and liver. The slight red tinge seen with Nile blue sulfate in the intra-alveolar cells and in certain of the largest adrenal cortical cells was suggestive of a mixture including triglycerides.

Descriptions of both Gaucher's and Niemann-Pick's diseases often mention the presence of diffuse hemosiderin deposits. In our case an intracellular pigment was prominent in most organs. This material did not stain for iron and thus could not be identified as hemosiderin. It stained as a fat with Sudan IV and Nile blue sulfate but was insoluble in alcohol and xylene. It had many of the histochemical characteristics of ceroid, but lacked acid-fastness which is regarded as essential for such identification. Since the pigment was found almost wherever there was lipid, their relation can scarcely be questioned, but the significance and nature of the pigment or of this relationship is unsolved.

Although the anatomical findings permitted a provisional diagnosis of Niemann-Pick's disease in an adult, a definitive diagnosis could not be made without chemical analysis. Therefore, portions of the lung, liver, and spleen were preserved in a frozen state for analysis of their lipid content.

Lipid Determinations in Lung, Liver, and Spleen

Procedure. Portions of the frozen tissues were thawed, and minced thoroughly with scissors. Samples were weighed into bottles for dry weight determination, and into Potter-Elvehjem homogenizer tubes for lipid extraction, which was carried out by homogenization[5] of the tissues in chloroform-methanol (2:1). The tissue suspensions were transferred quantitatively into 50 ml. volumetric flasks. Some fibrous material in the liver could not be completely homogenized; after removal of the suspension it was ground with fresh portions of solvent and finally transferred to the volumetric flask. The spleen also contained fibrous material which was difficult to homogenize, but eventually almost all of it was ground into fine suspension. The lung was homogenized rapidly and completely. The tissue suspensions stood in the tightly stoppered volumetric flasks for about 4 months until the analyses could be carried out.

Aliquots (15 ml. and 20 ml.) of the clear filtrates were washed by a modification of the procedure of Folch *et al.*[6] The 15 ml. aliquots were used for total lipid determination, and the 20 ml. aliquots, after dilution to various degrees according to the requirements of the methods, were analyzed for the following constituents: lipid phosphorus by the method of Sperry[7]; phosphosphingoside (sphingomyelin) phosphorus by the method of Schmidt *et al.*,[8] with the use of a modification of the Sperry method for the determination of the hydrolyzed phosphorus; total cholesterol and free cholesterol by the method of Sperry and Webb[9]; glycosphingosides (cerebrosides) by the method of Brand and Sperry[10]; and choline by an unpublished method of Sperry and Brand. All determinations were carried out in duplicate.

Results. The data are presented as percentages of dry tissue (Table III), of total lipids and total phosphatides (Table IV), and as ratios between certain lipids (Table V). Data on normal human tissue, obtained by the same methods, are not available for comparison. Somewhat similar procedures were used by Thannhauser *et al.*[11] in obtaining the values which are given in Table III for comparison. All of the lipid concentrations in the tissues of our patient are higher than the corresponding "normal" values, but the difference is small for liver

cephalin. Phosphosphingosides show by far the largest increases above normal in all three tissues.

The findings are of particular interest in their relation to reported analyses of tissues from patients with Niemann-Pick's disease. Comparison is difficult because the methods employed by us are different from those used by other investigators, most of whom have separated and isolated the lipids by procedures based on solubilities in different solvents. With painstaking work, pure lipids may be isolated from

TABLE III
Lipids in Liver, Spleen, and Lung

Constituent	Liver		Spleen		Lung	
	In dry tissue		In dry tissue		In dry tissue	
	Normal*	Our patient†	Normal*	Our patient	Normal*	Our patient
	%	%	%	%	%	%
Total lipid		36.0		33.3		41.6
Total phosphatide	9.1–11.1	25.7	5.5–11.3	22.7	6.0– 7.9	26.9
Choline-containing phosphatide	3.7– 6.7	20.5	3.9– 4.9	14.9	4.7– 6.0	22.9
Phosphosphingoside	0.3– 4.3	10.8	0.7– 1.0	7.6	0.9– 2.0	15.3
Phosphoglyceride	7.7–10.8	14.9	4.7–10.4	15.1	5.1– 6.5	11.6
Lecithin	3.3– 5.7	9.7	3.1– 4.0	7.4	3.8– 4.0	7.6
Cephalin	3.0– 5.4	5.2	1.6– 6.9	7.7	1.4– 2.6	4.0
Total cholesterol	2.0– 2.6	2.6	1.8– 2.4	3.8		6.0
Free cholesterol	0.4– 0.5	2.5	1.0– 1.1	3.6		5.8
Glycosphingoside		1.4		0.7		2.6
Undetermined lipid		6.3		6.2		6.2

* The normal values are the data of Thannhauser *et al.*[11,16,27] on human tissues.

† The duplicate determinations of dry weight of liver were in poor agreement: 27.4 and 28.7 per cent. Some uncertainty, therefore, applies to values in this column.

mixtures by solvent fractionation. It is doubtful that quantitative separations by such methods can be achieved, because the solubility differences of pure lipids are not complete, and because the solubility of one pure lipid is greatly modified by the presence of others.[12] It is for these reasons that in several papers data are given for concentrations of "crude" and "purified" sphingomyelin in spleen and liver from patients with Niemann-Pick's disease. Some investigators have reported the crude ether extract of acetone-dried tissue as phosphoglyceride, and the crude hot alcohol extracts as sphingomyelin. Such data can be accepted, if at all, only as rough approximations.

Although the calculation of the amounts of lipids from the quantitative determination of various constituents is also subject to several

sources of error, we have preferred to use it because we believe it gives a more accurate picture of the lipid distribution than do isolation procedures.

The large increases in phosphosphingosides (Table III) certainly

TABLE IV

Lipids in Liver, Spleen, and Lung; Percentages in Total Lipids and in Total Phosphatides for Each Organ

Constituent	Liver		Spleen		Lung	
	In total lipid	In total phosphatide	In total lipid	In total phosphatide	In total lipid	In total phosphatide
	%	%	%	%	%	%
Total phosphatide	71.4		68.0		64.6	
Choline-containing phosphatide	57.0	79.9	44.8	65.8	55.0	85.1
Phosphosphingoside	30.0	42.0	22.7	33.4	36.6	56.7
Phosphoglyceride	41.5	58.0	45.4	66.7	28.0	43.3
Lecithin	27.1	37.9	22.1	32.5	18.4	28.4
Cephalin	14.4	20.0	23.3	34.2	9.6	14.9
Total cholesterol	7.2		11.3		14.4	
Free cholesterol	6.9		10.7		13.8	
Glycosphingoside	4.0		2.2		6.2	
Undetermined lipid	17.4		18.5		14.8	

point to Niemann-Pick's disease, but large as the accumulations of this lipid were in the liver and spleen of our patient, they were considerably smaller than the concentrations of purified sphingomyelin which have been found by others in the tissues of infants with Niemann-Pick's disease: 15 to 25 per cent in the liver and 13 to 33 per cent in the spleen.[13-17] (A single value of 2.7 per cent, reported by Klenk[14] in a Niemann-Pick spleen, is omitted.) For reasons already discussed these may be regarded as minimal values. It may be concluded that the deposition of phosphosphingoside in the liver and spleen of our patient was less than half as great as the average found by others in the infantile form of Niemann-Pick's disease.

It is stated by some investigators that the concentrations of the phosphoglycerides, lecithin and cephalin, in the liver and spleen of Niemann-Pick patients is not increased above normal. Thannhauser[18] stated that lecithin is only slightly, and cephalin not at all, increased. Actually, a considerable proportion of the data which have been reported for these phosphatides presents values higher than the normal values of Thannhauser *et al.*[11] However, because of uncertainties concerning the methods used, a positive interpretation of the findings is

impossible. Chargaff,[17] on the basis of the ratio of amino-nitrogen to total nitrogen, found that about 70 per cent of the mono-aminophosphatides from a Niemann-Pick spleen consisted of cephalin. Most of the phosphoglyceride values from our patient are considerably higher than the normal concentrations with which they are compared.

Although little mention has been made of it, a marked deposition of cholesterol in liver and spleen appears to be a characteristic feature of Niemann-Pick's disease. Concentrations of 6.3 to 7.4 per cent in

TABLE V

Ratios Between Weights of Lipids in Lung, Liver, and Spleen

Lipids compared	Lung	Liver	Spleen
Free cholesterol/total cholesterol	0.96	0.96	0.94
Phosphosphingoside/lecithin	2.00	1.11	1.03
Glycosphingoside/phosphosphingoside	0.17	0.13	0.10

the liver and 1.5 to 8.8 per cent in the spleen[16,19-25] have been reported. The concentration of cholesterol found by most investigators in dry livers and spleens of normal humans and laboratory animals is about 1 per cent, though higher values, up to about 2 per cent, have occasionally been reported. The cholesterol concentrations in the tissues of our patient are undoubtedly abnormal, particularly so in the lung. The almost complete absence of cholesterol esters is notable. Usually an increase in combined cholesterol is largely responsible for abnormal accumulations of cholesterol in tissues. Results of determinations of cholesterol fractions in Niemann-Pick tissues have not been found in the literature.

Data on glycosphingoside (cerebroside) concentrations in Niemann-Pick tissues are not available, as far as we have been able to discover, but some investigators have stated that cerebroside was not found in liver or spleen. Our results indicate that considerable amounts of glycosphingoside were present in the tissues of our patient, but it should be noted that the "unhydrolyzed" blank titrations were much higher than we observed in the application of our method to brain lipids. As the results were otherwise consistent, we have no reason to doubt that they are accurate as to the amounts of glycosphingosides present.

It remains to compare our findings with the only data on record from adults with Niemann-Pick's disease. We are indebted to Professor P. Favarger of the University of Geneva, Switzerland, for pro-

viding us with a description of the method which he employed in carrying out the analyses, the results of which are reported by Pfändler[4] and by Dusendschon.[3] It was essentially the solvent fractionation procedure of Baumann, Klenk, and Scheidegger.[26] The findings are of interest in the present context, not only because they were obtained in adults, but also because they include the only recorded lipid analyses on Niemann-Pick lungs. The data are reproduced in Table VI because of their importance and because some of them are available only in

TABLE VI

*Lipids in Liver, Spleen, and Lung from Two Adults with Niemann-Pick's Disease**

Constituent	Liver		Spleen		Lung	
	Case 1	Case 2	Case 1	Case 2	Case 1	Case 2
	%	%	%	%	%	%
Total phosphatide (% of wet weight)	5.7	3.45	6.5	2.95	5.11	3.7
Sphingomyelin (crude)		30.1		36.9		34.0
Sphingomyelin (purified)		21.8		23.3		22.1
Cephalin-lecithin (phosphoglyceride)		2.65		8.28		2.67
Total cholesterol		3.8		4.6		7.6
Neutral fat		5.35		10.6		5.0

* The data are assembled from the paper of Pfändler[4] and the thesis of Dusendschon.[3] The total phosphatide values are percentages of wet weight; the others are percentages of dry weight. Analyses by Professor Favarger.

Dusendschon's thesis,[3] a copy of which was kindly sent us by Professor Favarger.

The only data available from their case 1 are of concentrations of total phosphatide in wet tissue. The interpretation of these values is open to question since the tissues had been fixed in formalin, but the considerably higher percentages in case 1 than in case 2 strongly suggest that the large accumulation of sphingomyelin in the tissues from case 2 was exceeded in case 1.

The almost identical concentrations of purified sphingomyelin in the three organs from case 2 are about in the middle of the range of concentrations which have been found in livers and spleens of Niemann-Pick infants, and are much higher than those which are obtained in our patient. The phosphoglyceride concentrations are much lower than the corresponding values from our patient, and two (liver and lung) are well below the normal concentrations of Thannhauser *et al.*[11] Lecithin and cephalin were not separately determined. The cholesterol concentrations are a little higher than we found, but are of the same order of magnitude. Professor Favarger stated (personal communica-

tion) that he did not find appreciable quantities of cerebrosides in any of the tissues which he analyzed. It is clear from the foregoing comparison that there are marked differences between the lipid concentrations found by Favarger in tissues from Pfändler's adult patient and those which we obtained in our patient. Only for cholesterol were the values approximately the same.

Discussion

The formulation of a final diagnosis for the case reported in this paper has proved impossible if one is to accept the rigorous definitions of the several lipidoses. The clinical findings are compatible with either Gaucher's or Niemann-Pick's disease. The anatomical study inclined us strongly toward the latter. The evidence for this provisional decision was presented first by the very diffuse involvement, second by the vacuolated or foamy cytoplasm and relative poverty of nuclei of the lipid-filled histiocytes, and third by such staining reactions as were noted in the Smith-Dietrich and Mallory aniline blue preparations. A histologic indication of the presence of a mixture of lipids was given by the different dye reactions in various areas. The nature of the alcohol-insoluble pigment remains unsolved. Hemosiderin deposits are described in the lipidoses, but since the material was iron negative in numerous preparations, it can hardly be considered as such. Regardless of its composition, the pigment is diagnostic of neither condition.

The myocardial amyloid deposits were of the idiopathic type and are regarded as being unrelated to the systemic lipidosis.

Can the chemical findings in our patient be correlated with the diagnosis of Niemann-Pick's disease? The answer is in the negative if our data and those which have been obtained in Niemann-Pick tissues are compared at their face value, without regard to differences in methods. Our results show a large increase above normal in all of the lipids measured except cephalin in the liver. In Niemann-Pick's disease only phosphosphingoside is considered by most investigators to be appreciably elevated. Actually, fairly large accumulations of other lipids, especially cholesterol, seem to be fairly well established by the published data, but these changes have been obscured, or perhaps minimized, in the eyes of those interpreting the data by the huge deposits of phosphosphingoside.

On the other hand, there is no doubt that phosphosphingoside was increased proportionally far more than any other lipid in the tissues of our patient. Even though the concentrations were much lower than those which are characteristic of Niemann-Pick's disease, this result

suggests that the lipidosis from which our patient was suffering was related to Niemann-Pick's disease, though not identical with it.

We are grateful to Dr. James I. Berkman and Dr. Harry M. Zimmerman for editorial assistance, to Dr. Leon Roizin for his studies on the microsolubility of tissue lipids in this case, and to Mr. Antol Herskovitz who prepared the photomicrographs.

REFERENCES

1. Roizin, L. Personal communication.
1a. Mejbaum, W. Über die Bestimmung kleiner Pentosemengen, insbesondere in Derivaten der Adenylsaüre. *Ztschr. f. physiol. Chem.*, 1939, **258**, 117–120.
2. Merklen, P., Waitz, R., and Warter, J. Maladie de Gaucher diagnostiquée par la ponction de la rate. *Ann. méd.* 1933, **33**, 97–123.
2a. Myers, B. Gaucher's disease of the lungs. *Brit. M. J.*, 1937, **2**, 8–10.
3. Dusendschon, A. Deux cas familiaux de maladie de Niemann-Pick chez l'adulte. Doctoral thesis, University of Geneva, 1946.
4. Pfändler, U. La maladie de Niemann-Pick dans le cadre des lipoïdoses. *Schweiz. med. Wchnschr.*, 1946, **76**, 1128–1131.
5. Potter, V. R., and Elvehjem, C. A. A modified method for the study of tissue oxidations. *J. Biol. Chem.*, 1936, **114**, 495–504.
6. Folch, J., Ascoli, I., Lees, M., Meath, J. A., and LeBaron, F. N. Preparation of lipide extracts from brain tissue. *J. Biol. Chem.*, 1951, **191**, 833–841.
7. Sperry, W. Electrophotometric microdetermination of phosphorus in lipide extracts. *Indust. & Engin. Chem. (Anal. Ed.)*, 1942, **14**, 88–90.
8. Schmidt, G., Benotti, J., Hershman, B., and Thannhauser, S. J. A micromethod for the quantitative partition of phospholipide mixtures into monoaminophosphatides and sphingomyelin. *J. Biol. Chem.*, 1946, **166**, 505–511.
9. Sperry, W. M., and Webb, M. A revision of the Schoenheimer-Sperry method for cholesterol determination. *J. Biol. Chem.*, 1950, **187**, 97–106.
10. Brand, F. C., and Sperry, W. M. The determination of cerebrosides. *J. Biol. Chem.*, 1941, **141**, 545–553.
11. Thannhauser, S. J., Benotti, J., Walcott, A., and Reinstein, H. Studies on animal lipids. XV. The lecithin, cephalin, and sphingomyelin content of normal human organs. *J. Biol. Chem.*, 1939, **129**, 717–719.
12. Folch-Pi, J., and Sperry, W. M. Chemistry of the lipids. *Ann. Rev. Biochem.*, 1948, **17**, 147–168.
13. Klenk, E. Über die Natur der Phosphatide und anderer Lipoide des Gehirns und der Leber bei der Niemann-Pickschen Krankheit. *Ztschr. f. physiol. Chem.*, 1935, **235**, 24–36.
14. Klenk, E. Beiträge zur Chemie der Lipoidosen. 3. Mitteilung. Niemann-Picksche Krankheit und amaurotische Idiotie. *Ztschr. f. physiol. Chem.*, 1939–40, **262**, 128–143.
15. Menten, M. L., and Welton, J. P. Lipid analysis in a case of Niemann-Pick disease. *Am. J. Dis. Child.*, 1946, **72**, 720–727.
16. Thannhauser, S. J. Lipidoses. Oxford University Press, New York, 1950, ed. 2, p. 546.
17. Chargaff, E. A study of the spleen in a case of Niemann-Pick disease. *J. Biol. Chem.*, 1939, **130**, 503–511.
18. Thannhauser, S. J. Lipidoses. Oxford University Press, New York, 1950, ed. 2, p. 544.

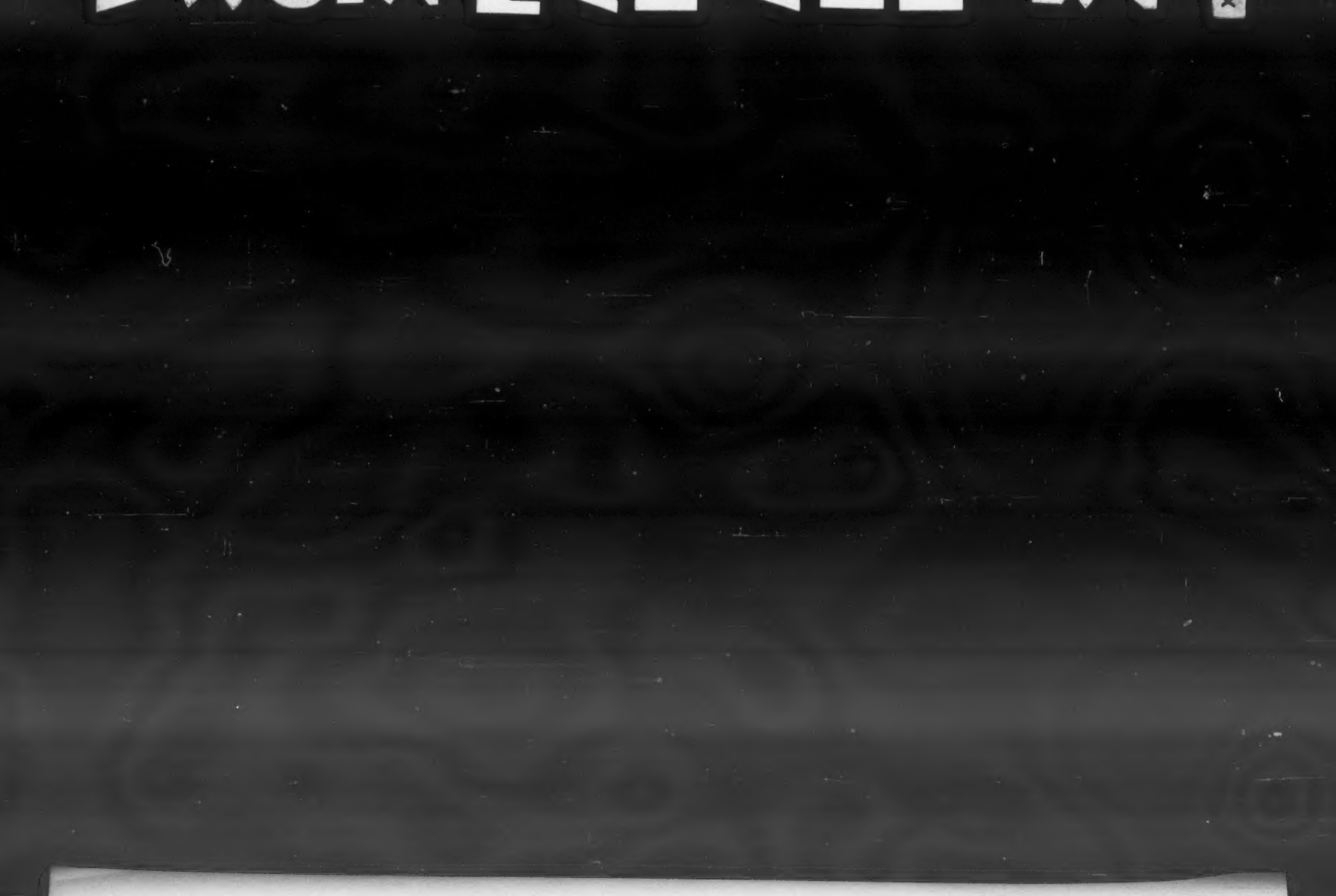
19. MacFate, R. P. The chemical analysis of liver and spleen from a case of lipoid histiocytosis (Niemann-Pick's disease). *Arch. Path.*, 1928, 6, 1054–1057.
20. Epstein, E., and Lorenz, K. Die Phosphatidzellverfettung in Gehirn, Leber und Milz bei Niemann-Pickscher Krankheit. *Ztschr. f. physiol. Chem.*, 1932, 211, 217–230.
21. Teunissen, P. H. Beitrag zur Kenntnis der Chemie der Lipoidosis phosphatidica. *Ztschr. f. physiol. Chem.*, 1937, 248, 142–150.
22. Bloom, W., and Kern, R. Spleens from Gaucher's disease and lipoid-histiocytosis. *Arch. Int. Med.*, 1927, 39, 456–461.
23. Brahn, B., and Pick, L. Zur chemischen Organanalyse bei der lipoidzelligen Spleno-Hepatomegalie Typus Niemann-Pick. *Klin. Wchnschr.*, 1927, 6, 2367–2369.
24. Epstein, E., and Lorenz, K. Zur Chemie der Gewebseinlagerungen bei einem Falle von Schüller-Christianscher Krankheit. *Ztschr. f. physiol. Chem.* 1930, 190, 44–50.
25. Epstein, E., and Lorenz, K. Die Phosphatidzellverfettung der Milz bei Niemann-Pickscher Krankheit verglichen mit der Lipoidchemie des Morbus Gaucher und der Schüller-Christianschen Krankheit. *Ztschr. f. physiol. Chem.*, 1930, 192, 145–170.
26. Baumann, T., Klenk, E., and Scheidegger, S. Die Niemann-Picksche Krankheit. Eine klinische, chemische und histopathologische Studie. *Ergebn. d. allg. Path. u. path. Anat.*, 1936, 30, 183–323.
27. Thannhauser, S. J. Lipidoses. Oxford University Press, New York, 1950, ed. 2, p. 470.

[*Illustrations follow*]

LEGENDS FOR FIGURES

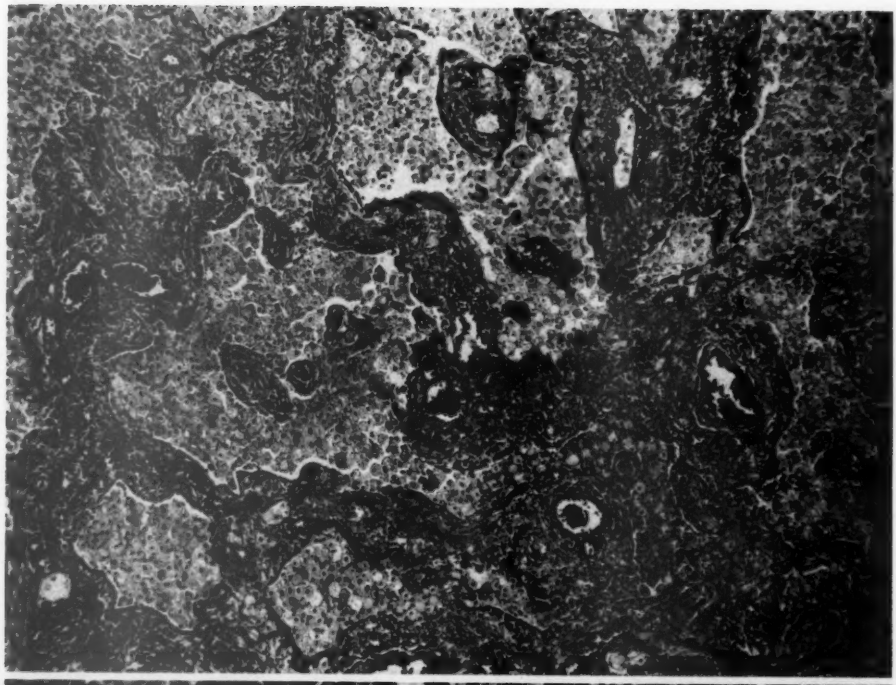
FIG. 1. Lung. The alveoli are filled by lipid-containing histiocytes, and the septa are sclerotic. Hematoxylin and eosin stain. × 85.

FIG. 2. Lung. The alveolar lining cells and foam cells are apparent in this section. Hematoxylin and eosin stain. × 250.

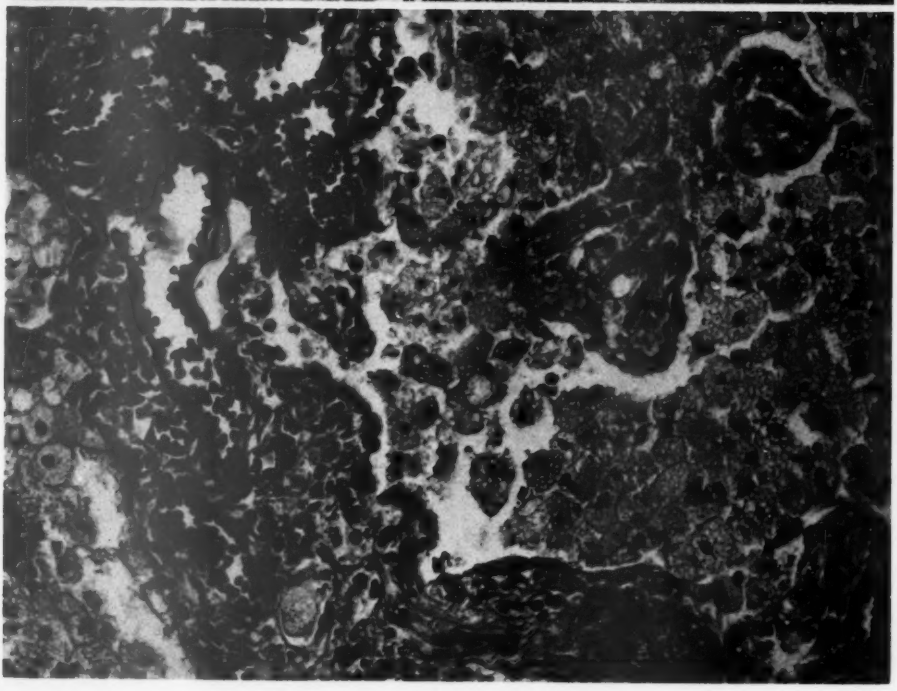


1

2



1

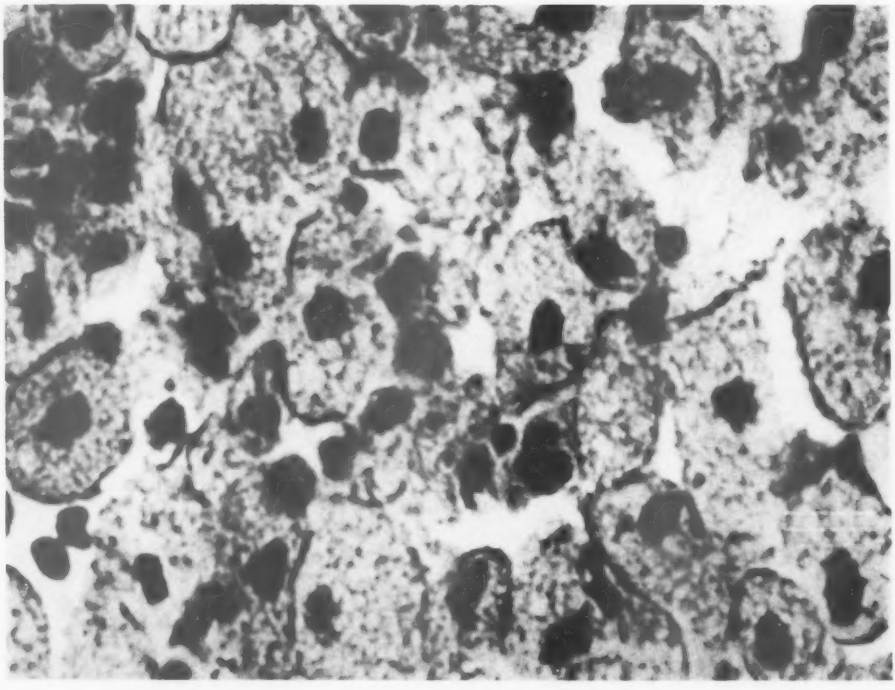


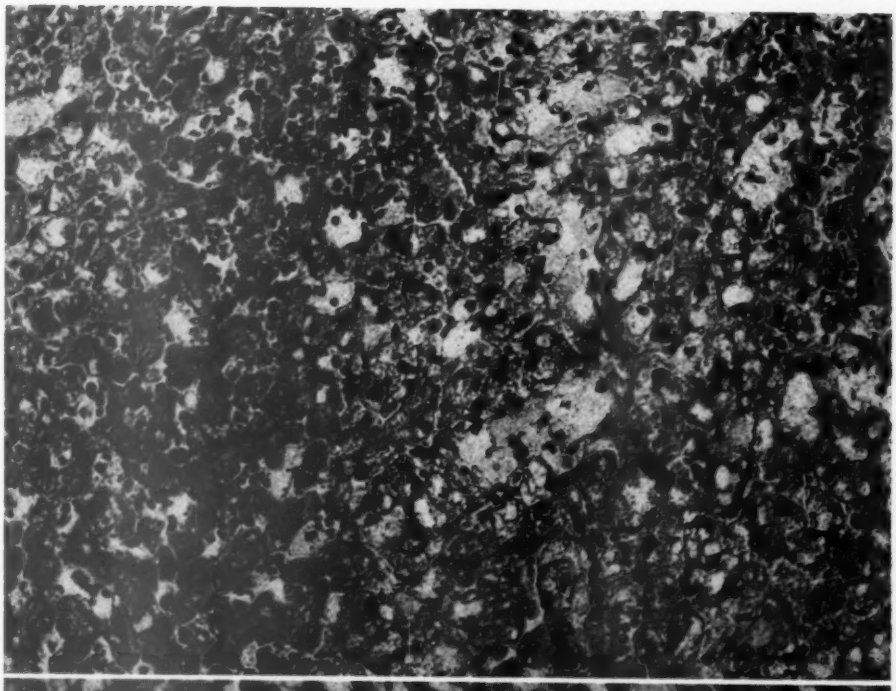
2

FIG. 3. Lung. These intra-alveolar histiocytes are similar to those found in most of the other tissues. Mallory's aniline blue stain. × 1200.

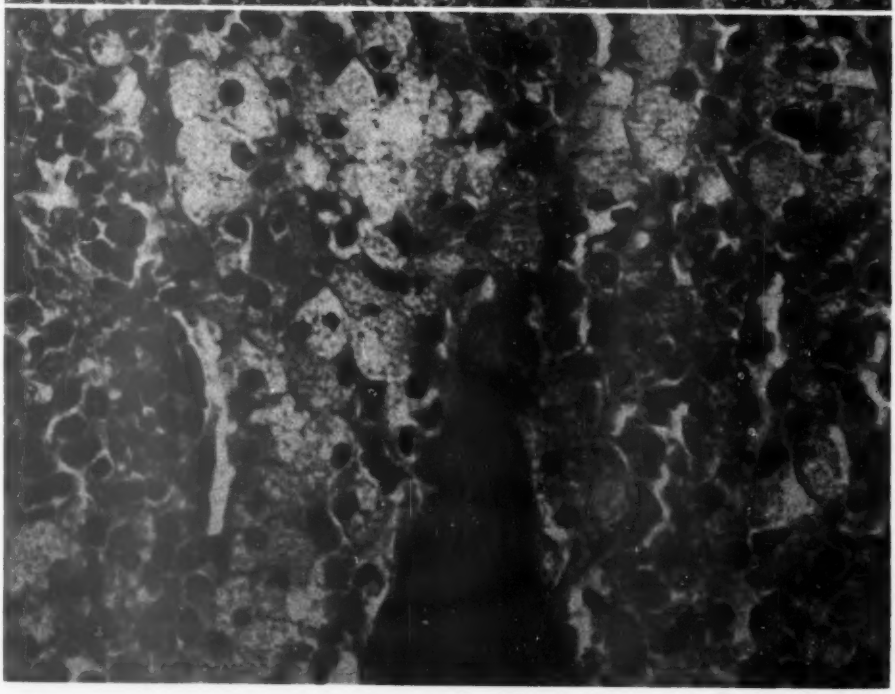
FIG. 4. Liver. The Kupffer cells are massively enlarged. Hepatic cord cells are less often ballooned, but are commonly vacuolated. Hematoxylin and eosin stain. × 250.

FIG. 5. Spleen. Enlarged, foamy histiocytes have replaced most of the normal splenic elements. Hematoxylin and eosin stain. × 540.





4

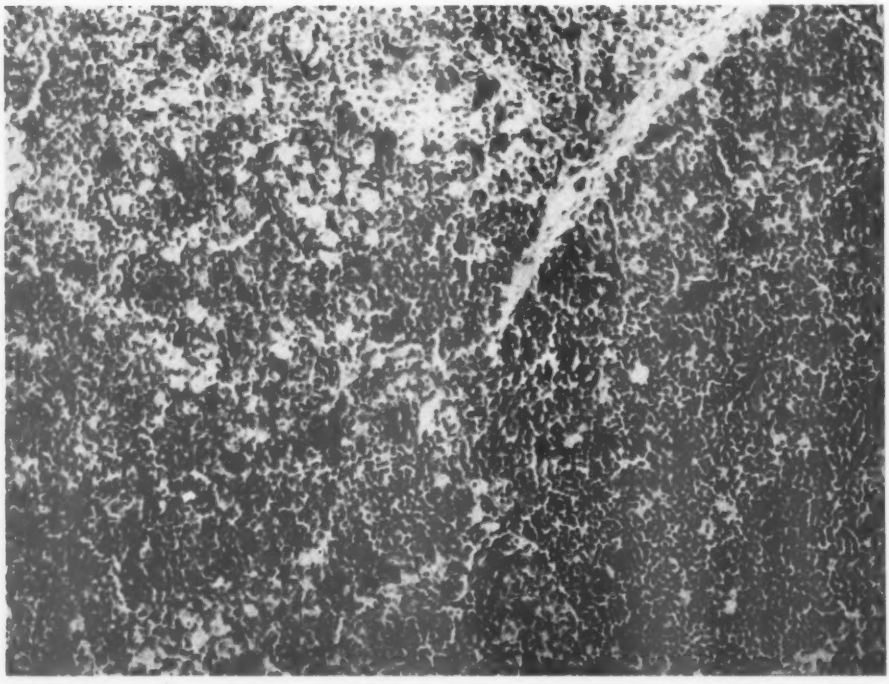


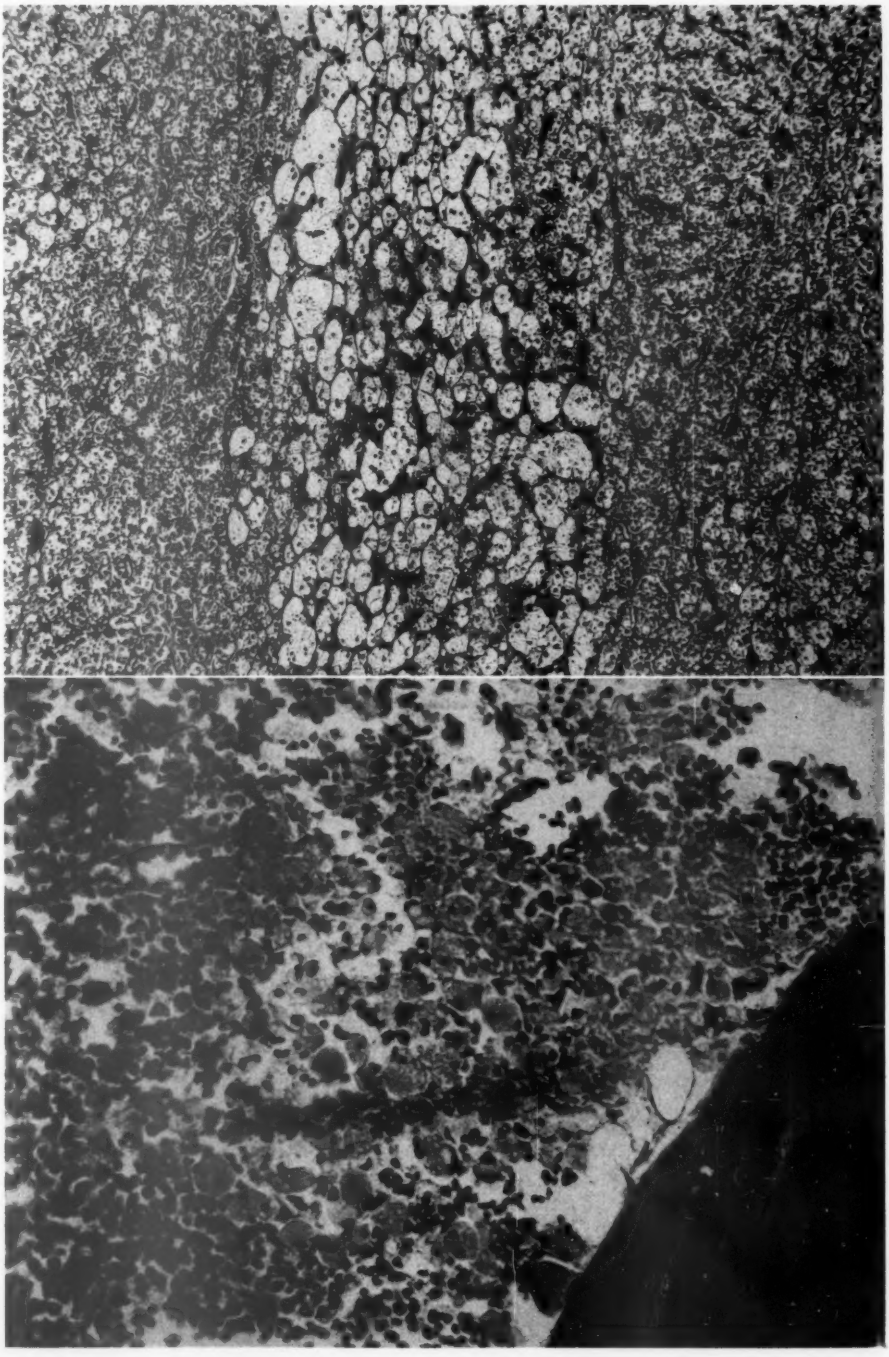
5

FIG. 6. Lymph node. Groups of histiocytes are seen scattered in otherwise normal lymphoid tissue. Hematoxylin and eosin stain. × 150.

FIG. 7. Adrenal cortex. Most cortical cells are moderately ballooned with lipid. A streak of very large, lipid-containing cells is seen in the center. Hematoxylin and eosin stain. × 85.

FIG. 8. Vertebral bone marrow. There is a large central focus of histiocytes surrounded by hyperplastic marrow. The trabecula is normal. Hematoxylin and eosin stain. × 300.





GENERALIZED SIDEROSIS WITH FIBROSIS OF LIVER AND PANCREAS IN COOLEY'S (MEDITERRANEAN) ANEMIA

With Observations on the Pathogenesis of the Siderosis and Fibrosis*

John T. Ellis, M.D., Irving Schulman, M.D., and Carl H. Smith, M.D.
(From the Departments of Pathology and Pediatrics, the New York Hospital-Cornell Medical Center, New York, N.Y.)

Iron pigment has been found regularly in the organs of 10 cases of Cooley's anemia examined during infancy and early childhood by previous workers, but visceral fibrosis has not been observed.[1-6] In the present work, fibrosis of the liver and pancreas, together with extensive siderosis of these and other organs, was encountered in 11 of 13 cases of Cooley's anemia that were examined anatomically after having been under clinical observation for periods of 2 to 27 years. The anatomical changes were similar to, if not identical with, those that characterize idiopathic hemochromatosis. Similar observations have been made recently in 2 other cases in which therapy prolonged the lives of children with Cooley's anemia.[7,8] The visceral changes as observed in the 13 cases will be described in this paper and correlated with certain clinical observations. Four illustrative cases will be reported in detail. The findings throw light on the origin of the iron pigment comprising the siderosis, and they bear on the question whether excessive deposits of iron, present for long periods of time, may lead to cirrhosis of the liver and pancreatic fibrosis.

Materials and Methods

Six cases of Cooley's anemia were examined post mortem (nos. 1, 2, 5, 8, 12, and 13), and specimens of liver were taken for biopsy at the time of splenectomy from 7 cases. In addition to routine histologic preparations stained with hematoxylin and eosin, sections were stained with Gomori's ferrocyanide method for hemosiderin, while Masson's trichrome and Mallory's aniline blue stains were employed to identify the collagen of connective tissue. The visceral siderosis and fibrosis were graded on the basis of histologic observations according to criteria that will be given in the body of the paper. Chemical analyses

* Presented at the Fiftieth Annual Meeting of the American Association of Pathologists and Bacteriologists, St. Louis, April 2, 1953.
Clinical studies carried out in the Department of Pediatrics were aided by grants from the United Public Health Service, the Children's Blood Foundation, Inc., and Mead Johnson Co.
Received for publication, July 15, 1953.

for iron were performed on samples of the liver in 2 cases (nos. 1 and 6) according to the method described by Finch *et al.*[9]

Character of the Fibrosis and Siderosis

A diffuse, finely nodular cirrhosis with marked hepatomegaly was found in 3 of the 6 cases studied post mortem (nos. 1, 2, and 8), the livers being firm, having a finely nodular surface, cutting with increased resistance, and on section having small nodules surrounded by depressed gray fibrous tissue. In these 3 cases with severe fibrosis the fibrous bands linked the portal areas with one another and with the hepatic veins, while the liver cords within the lobules were arranged irregularly and the central vein was often eccentrically placed (Fig. 1). Similar but less extensive changes were found in the 8 remaining cases. The extent of the fibrosis in these cases will be indicated further on. Proliferation of bile ducts was in general inconspicuous and infiltration with chronic inflammatory cells was minimal. The connective tissue consisted of moderate numbers of fibroblasts and much collagenous tissue. Focal areas of regeneration were noted microscopically in 2 cases and in one of these there was a circumscribed nodule of regeneration 3 cm. in diameter. Necrosis or degenerative changes were not noted except in one case in which the hepatic cells contained many lipid vacuoles.

Diffuse proliferation of the interlobular and intralobular connective tissue of the pancreas was present in varying degrees in 4 of the 6 necropsied cases. In the 2 cases with severe fibrosis, one of which was associated with diabetes mellitus (case 1), fibrous bands of varying widths completely surrounded, and extended into, masses of acinar and islet cells, dividing the organ into nodules of varying sizes (Fig. 3). The fibrous bands contained mature collagenous tissue as well as fibroblasts and a few chronic inflammatory cells. No abnormalities of the ducts were present. In 2 other cases also fibrosis was present, though it was less extensive, and there were randomly scattered focal areas in which fibrous tissue isolated individual acini (Fig. 7).

Hepatic siderosis, as determined by microscopic examination of tissues stained specially for iron, was observed in all 13 cases. The iron pigment was distributed diffusely throughout the cells of the liver lobules, except in the one case with minimal involvement in which the peripheral portions of the lobules were more heavily pigmented, and in two of the cirrhotic livers in which regenerating tissue was much less extensively pigmented than was adjacent liver parenchyma. In most cases the hemosiderin granules filled the cytoplasmic spaces of

the hepatic cells, though in some it was concentrated around the nuclei of the hepatic cells, or distributed more scantily through the cytoplasm, or concentrated on the bile-capillary side of the cells. Large amounts of pigment were likewise present in the Kupffer cells, in the epithelial cells of some of the smaller bile ducts, and in macrophages and connective tissue cells in the portal areas and fibrous bands of the cirrhotic livers (Figs. 2 and 6). The walls of blood vessels in the portal areas often contained large amounts of iron pigment,* and this was sometimes present in focal areas in the walls of bile ducts also, though the epithelium of the latter was usually devoid of pigment.

Extensive and diffuse deposits of hemosiderin were present in the pancreas in 4 cases in which hepatic siderosis was marked, while no pigment was found in the pancreas in 2 cases in which hepatic siderosis was relatively slight. The acinar cells of the involved pancreas were heavily pigmented, often to such an extent as to obscure the cellular outline (Fig. 4), though in less heavily pigmented cells the hemosiderin was concentrated between the nucleus and the alveolar lumen (Fig. 8). The islets of Langerhans likewise contained hemosiderin, though usually less than the acinar cells, and some of the cells in each islet usually were spared (Figs. 4 and 8). Lesser amounts of pigment were found in macrophages and connective tissue cells of the interstitium and in those of fibrous bands. The epithelial cells of the small and medium-sized pancreatic ducts contained irregularly distributed hemosiderin, the pigment often sparing individual cells and sometimes the cells of entire ducts (Fig. 4).

In addition to the hemosiderin deposits in the liver and pancreas, pigment was found in lymph nodes, spleen, myocardium, renal tubules, thyroid, adrenal, and parathyroid glands, pituitary body, mucosa of the stomach and ileum, prostate, seminal vesicle, epididymis, testis, and bronchial glands in a distribution similar to that reported previously.[1,2] Iron pigment was not demonstrated in the epidermis of 5 cases in which skin was studied. The distribution of pigment in these cases of Cooley's anemia, it may be noted here, was quite similar to that previously observed by Whipple and Bradford,[1,2] who first directed attention to the similarity of the pigmentation in this disease and that in idiopathic hemochromatosis.

In 3 of the cases of Cooley's anemia here reported, the cirrhosis of the liver and fibrosis of the pancreas together with the widespread

* Hemofuscin pigment was present in large amounts in the liver of case 7, occurring principally in the walls of the blood vessels and small bile ducts. This pigment was not found in any of the other 11 cases.

visceral siderosis—including involvement of the hepatic cord cells and epithelium of some of the bile ducts, acinar and islet cells of the pancreas, and connective tissue cells of the fibrous bands in liver and pancreas—comprised anatomical changes identical with those that characterize idiopathic hemochromatosis,[10,11] transfusion hemosiderosis with fibrosis,[12-17] and nutritional cytosiderosis with fibrosis,[18,19] while in 8 additional cases the changes were similar though less extensive. As will be brought out later, the quantity of iron in the liver, as determined chemically in 2 of the cases studied here, also falls within the range reported in idiopathic hemochromatosis.

Gradations of Siderosis and Fibrosis in Relation to Iron Given Therapeutically and to Duration of the Disease

To learn whether any correlations exist between the degrees of siderosis and fibrosis on the one hand and the amount of iron given therapeutically on the other, the pertinent observations in the 13 cases were brought together in Table I. It may be seen that varying degrees of hepatic siderosis were found in all 13 cases, and that pancreatic siderosis was present in 4 of the 6 cases examined, being absent in 2 cases in which hepatic siderosis was relatively slight. In general, the histologic observations indicate that the degree of hepatic and pancreatic siderosis paralleled the degree of fibrosis of these organs.

Relations of Therapeutically Administered Iron and Siderosis. From Table I it may be seen that the iron contained in the transfused blood and that administered orally could not have been wholly responsible for the hemosiderosis. For example, one patient (case 2) did not receive iron by mouth and was given only 0.5 gm. of iron by transfusion —and this on the day of death—yet manifested extreme hepatic and pancreatic siderosis at necropsy, this being fully as extensive, as judged histologically, as that seen in any of the other cases. A second patient (case 7), who received 6.3 gm. of iron in repeated blood transfusions and none by mouth or otherwise, likewise had siderosis that was extreme and equal to or exceeding that found in 7 other cases that had received much larger amounts of iron by way of repeated transfusions (cases 3, 4, 6, 8, 9, 10, and 11, which had been given 51.0, 49.4, 27.6, 16.0, 19.6, 25.6, and 28.8 gm. of transfused iron, respectively). In a third patient (case 1) chemical analysis showed that the 3600 gm. liver contained 27.36 gm. of iron, while only 6.6 gm. of iron had been given by repeated transfusions, the finding being similar to that noted by others in so-called transfusion siderosis in which more iron was present in the liver than had been given in the transfusions.[15,16]

TABLE I

Gradations of Siderosis and Fibrosis in the 13 Cases in Relation to Iron Given Therapeutically and to Duration of the Disease

Case no.	Sex	Age	Iron given therapeutically*	Siderosis†		Fibrosis‡	
				Liver	Pancreas	Liver	Pancreas
			By transfusion, gm.				
1	M	20	6.6	++++§	++++	++++	++++
2	F	27	0.5	++++	++++	++++	+++
3	F	15	51.0	++++		+++	
4	M	13	49.4	++++		+++	
5	M	6	4.2	++++	+++	+++	++
6	M	6	27.6	++++‖		+++	
7	F	3	6.3	++++		+++	
8	M	7	16.0	+++	+++	++++	++
9	M	7	19.6	+++		+	
10	M	7	25.6	++		+++	
11	F	13¶	28.8	++		++	
12	M	4	1.8	++	o	o	o
13	M	2	1.2	+	o	o	o

* In addition to the transfused iron as tabulated above, case 1 was given 150 mg. of elemental iron per day orally for 4 years; case 5, 275 mg. per day for 5½ years; case 9, 150 mg. per day for 2 years; case 13, 200 mg. per day for 15 months. In calculating the amount of transfused iron given, it was assumed that 1 liter of transfused blood contained 0.5 gm. of iron.

† Siderosis of liver:

++++ Extreme pigmentation of hepatic cord cells throughout the lobules, and in Kupffer cells; also in macrophages and connective tissue cells of the fibrous tissue, and in some of the bile ducts (Fig. 2).

+++ Lesser degree of pigmentation of same structures (Fig. 6).

++ Many small granules of pigment in majority of hepatic cord cells and many large granules in Kupffer cells.

\+ Fine granules of pigment in hepatic cord cells in periphery of liver lobule and in some Kupffer cells.

Siderosis of pancreas:

++++ Extreme pigmentation of acinar cells, macrophages, and epithelial cells of pancreatic ducts; moderate pigmentation of connective tissue and islets of Langerhans (Fig. 4).

+++ Less extensive pigmentation of same structures (Fig. 8).

‡ Fibrosis of liver:

++++ Broad bands of fibrous tissue completely encircling the liver lobules and dividing them into nodules of irregular size (Figs. 1 and 5).

+++ Narrower bands of fibrous tissue almost completely encircling liver lobules.

++ Narrow bands of fibrous tissue in portal areas with slight extension around liver lobules.

\+ Early fibrous tissue proliferation in portal areas with slight extension around liver lobules.

Fibrosis of pancreas:

++++ Broad bands of fibrous tissue dividing parenchyma into nodules of irregular size (Fig. 3).

+++ Narrower bands of fibrous tissue dividing parenchyma into nodules of varying sizes.

++ Diffuse fine interlobular and intralobular fibrosis (Fig. 7).

§ Chemical analysis:

7.6 mg. iron/gm. liver—total iron in liver (3600 gm.) = 27.36 gm.

‖ Chemical analysis:

8.1 mg. iron/gm. liver.

¶ Onset of symptoms at age 7, all other cases having onset during first year of life.

Relation of Duration of Disease to Fibrosis. As mentioned, hepatic and pancreatic fibrosis was not observed in the infants and young children with Cooley's anemia studied post mortem some years ago,[1-6] whereas it was conspicuous in the patients of the present study in whom the duration of the disease was much longer, owing presumably to more adequate therapy. This was true in 2 other cases of longer duration recently studied elsewhere.[7,8] Can it be inferred that the fibrosis is related to the duration of the disease? The findings of Table I provide evidence on this point. For practical purposes the age of the patient may be considered approximately equal to the duration of the disease, for in 12 of the 13 cases symptoms and signs of the disease led to its diagnosis during the first year of life. In the remaining case, the delayed clinical onset at age 7 may readily be taken into account. From Table I it may be seen that hepatic fibrosis was present in all 10 patients who were 6 years of age or older, the more extensive examples of hepatic and pancreatic fibrosis being found in the older individuals, while fibrosis was not present in these viscera in 2 of the 3 patients under 6 years of age. These findings suggest a relationship between duration of the disease and the fibrosis. The following observations, however, provide contrary evidence. In case 7 (age 3), for example, hepatic fibrosis, as judged histologically, equalled or exceeded that found in 7 other patients ranging from 6 to 15 years of age, while in case 8 (age 7), hepatic fibrosis equalled or exceeded that found in any of the other remaining 12 patients ranging to 27 years of age. These observations indicate that some factor or factors other than duration of the disease may have been responsible for the fibrosis in the cases here reported.*

Details of Four Illustrative Cases

Case 1

Extensive visceral siderosis and fibrosis of the liver and pancreas with associated diabetes mellitus in a 20-year-old Italian male who had been given intermittent transfusions (total 6.6 gm. of iron). Chemical analyses disclosed that the liver contained 27.36 gm. of iron.

The patient, an Italian male, 20 years of age, was first admitted to the New York Hospital on December 31, 1932, at 8½ months of age for intermittent upper respiratory infections since birth, pallor, and failure to gain weight. The skin was waxy and pale and there were bossing of the skull, a systolic heart murmur, and marked enlargement of the liver and spleen.

---

* After completion of the manuscript an additional case was studied by biopsy of the liver at the time of splenectomy, and provides additional evidence of the relation of duration of disease to fibrosis. The patient, an Italian male, 17 years of age, had been given periodic blood transfusions since the age of 9 months (total, 41.8 gm. of iron). The liver showed 2 plus fibrosis and 4 plus siderosis as graded by the criteria used in Table I.

The blood count showed: red blood cells, 1.27 millions per cmm.; hemoglobin, 4.5 gm. per 100 cc.; white blood cell count, 9450, of which 27 per cent were polymorphonuclear leukocytes, 7 per cent monocytes, and 66 per cent lymphocytes; nucleated red blood cells, 94 per cmm.; platelets, 125,000 per cmm.; reticulocytes, 20.6 per cent. The red blood cells showed anisocytosis, poikilocytosis, and microcytosis; the icterus index was 9.3; fecal urobilinogen, 9.04 gm. in 24 hours; red blood cell fragility test revealed beginning hemolysis at 0.46 per cent sodium chloride and ending at 0.32 per cent; bleeding time, 2 minutes; clotting time, 2½ minutes; clot retraction, normal. A gastric analysis revealed no free hydrochloric acid to histamine. Roentgenograms of the skull showed striations between the tables in the posterior portions, and in the long bones there was thinning of the cortex and increased trabeculation. Those of the chest revealed the heart to be globular and slightly enlarged. The patient was treated with transfusions of whole blood and discharged.

From June, 1933, to June, 1934, the patient was admitted to the hospital three times and treated for anemia with transfusions and various iron-containing preparations. Because of low hemoglobin levels, the patient was transfused weekly from December, 1933, to July, 1934, when splenectomy was performed. After splenectomy there was marked improvement and the red cell count was maintained at about 3.5 millions per cmm. without transfusions. In November, 1934, roentgenograms of the skull showed marked improvement in respect to previously reported changes. The patient remained well without transfusions and was seen in 1936 and in 1939, when the hemoglobin was 8.8 gm. per 100 cc. In April, 1942, hypospadias was corrected and at that time the hemoglobin was 7.5 gm., no transfusions having been given since splenectomy in 1934. Two transfusions were given at this time.

The patient remained well until September 27, 1945, when he was admitted for *Bacillus coli* meningitis which responded to penicillin and sulfadiazine. During this admission the hemoglobin ranged from 7 to 8 gm. and two transfusions were given. In 1949, two, in 1950, five, and in 1951, two transfusions were given for anemia, usually with hemoglobin levels ranging from 5 to 8 gm. per 100 cc. On June 25, 1952, the patient entered the hospital because of dizziness, orthopnea, dyspnea, and ankle edema. Examination revealed marked respiratory distress, enlargement of the liver, pitting edema of the lower extremities, and a systolic thrill and murmur. Laboratory examinations were as follows: hemoglobin, 3.5 gm. per 100 cc.; blood urea nitrogen, 14 mg. per cent; CO_2 combining power, 24 mEq./l.; total protein, 6.7 gm.; albumin, 3.6 gm.; globulin, 3.1 gm. A glucose tolerance test revealed a fasting blood sugar of 112 mg. per cent; 30 minute sample, 200 mg. per cent; 1 hour sample, 250 mg. per cent; 2 hour sample, 243 mg. per cent; 3 hour sample, 104 mg. per cent; and 4 hour sample, 63 mg. per cent. The patient was treated with transfusions, digitalis, and quinidine and was later discharged as improved. He entered the hospital for the last time on September 11, 1952, because of chest pain and diabetic acidosis. The hemoglobin at that time was 10 gm. per 100 cc; the urine showed 3 plus acetone and diacetic acid and 4 plus sugar; blood sugar, 456 and 634 mg. per cent; and blood urea nitrogen, 15 mg. per cent. Death occurred 2 days later.

Necropsy was performed 2½ hours after death. The liver weighed 3600 gm., was dark brown, and had a finely nodular surface. The parenchyma cut with increased resistance and was divided into irregular 3 to 5 mm. nodules by fine, gray, depressed bands of fibrous tissue. There were numerous small nodules of parenchymatous regeneration 0.5 to 1 cm. in diameter, and deep in the right lobe there was a single 3 cm., spherical, circumscribed, and encapsulated nodule which

was less heavily pigmented than the remainder of the liver. The pancreas weighed 130 gm., was dark brown, and increased in firmness. The cervical, hilar, mediastinal, periaortic, peripancreatic, mesenteric, pelvic, and inguinal lymph nodes were brown and enlarged up to 3 by 3 by 2.5 cm. The myocardium was dark brown and firm. The Prussian-blue reaction for iron on the liver, pancreas, lymph nodes, and heart was strongly positive. The external genitalia were small and the testicles were atrophic, measuring 2.3 by 1.5 by 1 cm., and very firm. The vertebral bone marrow was abundant and dark red-brown. The spleen was surgically absent.

Microscopically, broad bands of collagenous tissue joined the portal tracts with each other and with the hepatic veins and divided the parenchyma into nodules of varying sizes (Fig. 1). Within the nodules the central veins, if present, were often eccentrically placed and there were many nodules in which the liver cords had lost their orderly radiating arrangement. In the fibrous bands there were many fibroblasts and mature collagenous fibers, and a scanty infiltration of lymphocytes. Proliferation of small bile ducts was minimal. Extreme hemosiderosis was present diffusely throughout the liver lobule; however, the hepatic cord cells in the outer two thirds of the lobule usually contained the greater amount of pigment. The iron pigment often entirely filled the cytoplasm of the cell; however, in cells with less pigment it was usually concentrated on the bile-capillary side of the cell. Pigmentation was usually of a much lesser degree in the regenerated nodules than in adjacent hepatic cells. Hemosiderin was present in large amounts in Kupffer cells and also in the connective tissue fibers and in macrophages of the fibrous bands (Fig. 2). Some of the collagenous fibers, in specially stained preparations, were shown to contain both iron pigment and calcium salts. The fibrous bands also contained a number of discoid and lamellated masses of hemosiderin admixed with calcium salts and surrounded by multinucleated giant cells. The epithelial cells of many smaller bile ducts contained hemosiderin, although some lacked it. In some instances, the cells forming the walls of the bile ducts and small blood vessels contained much hemosiderin as did the endothelial cells and basement membranes.

The pancreatic alterations comprised an extensive diffuse interlobular and intralobular fibrosis, dividing the parenchyma into nodules of varying sizes (Fig. 3). The fibrous bands contained much mature collagen, a moderate number of fibroblasts, many pigmented macrophages, and a few scattered lymphocytes. Although fibrous tissue often encircled islets of Langerhans, no degenerative changes or fibrosis occurred within the islets. The pancreatic ducts appeared normal.

Hemosiderosis of the pancreas was diffuse, the pigment being found in greatest concentration in the acinar cells where it often entirely filled the cytoplasm (Fig. 4). The iron pigment usually was scattered diffusely throughout the cytoplasm, often obscuring the cellular outline, but in less pigmented cells it was concentrated between the nucleus and the alveolar lumen. Lesser amounts were seen in macrophages and in connective tissue cells and fibers of the interstitium. The epithelium of the small and medium-sized pancreatic ducts contained irregularly distributed hemosiderin pigment (Fig. 4). The islets of Langerhans contained pigment, though often in slightly lesser degree than in the acinar cells (Fig. 4).

Hemosiderin was found in large amounts in the epithelial cells of the thyroid and parathyroid glands, zona glomerulosa of the adrenal gland, distal convoluted tubules of the kidney, bronchial glands, prostate, seminal vesicle, and epididymis; in macrophages in sinusoids of lymph nodes, bone marrow, and lung; and in the myocardial fibers.

The germinal epithelium of the testes was atrophic; the tubules were lined largely by Sertoli cells and surrounded by thick collars of fibrous tissue.

Other findings consisted of acute bronchopneumonia, chronic rheumatic heart disease with slight stenosis of the mitral valve, and bilateral pleural effusions.

Case 2

Extensive hepatic and pancreatic siderosis and fibrosis in a 27-year-old Italian female who had not received blood transfusions until the day of death and who had not been given oral iron preparations.

The patient, an Italian female, first came under observation in 1913 at the age of 9 years because of chills, fever, weakness, and enlargement of the liver and spleen. Splenic enlargement had been noted at the age of 1 to 2 years, and darkening of the skin was apparent at the age of 8. The red blood cell count was 2,336,000 per cmm.; hemoglobin, 4.0 gm. per cent; white blood cells, 8100 per cmm. The red cells showed poikilocytosis, anisocytosis, polychromatophilia, and 66 per cent of the nucleated cells were normoblasts. One year later, in 1914, splenectomy was performed and was followed by clinical improvement. In 1920 at the age of 16 the patient was treated for femoral phlebitis, and at that time the hemoglobin was 7.2 gm. per cent with blood findings similar to those in 1913. The patient remained well and was not observed again until 1926 when she was admitted for anemia during pregnancy. Later she was delivered of a deadborn fetus 2 weeks before term. In 1929 a second pregnancy was terminated for toxemia. From 1927 to 1931 hemoglobin levels ranged from 4 to 6 gm. per cent. In January, 1932, the patient, then 27 years of age, was admitted for severe prostration and hepatic enlargement. Examination revealed hemic heart murmurs, râles at the lower base of the left lung, distention of the neck veins, respirations 29 per minute, and blood pressure of 84/22 mm. of Hg. The red blood cell count was 810,000 per cmm.; hemoglobin, 2.5 gm. per cent; white cell count, 18,310 per cmm., with 75 per cent polymorphonuclear leukocytes, 25 per cent lymphocytes; nucleated red blood cells, 16,238 per cmm. The patient was given one transfusion and died 10 hours later.

Necropsy was performed 11 hours post mortem and revealed an enlarged, brown, smooth liver weighing 2200 gm. On section the parenchyma was uniformly brown with indistinct lobular markings. The gallbladder was distended with yellow bile, contained a round black stone, 2 cm. in diameter, and five similar smaller stones, and was adherent by fibrous adhesions to the transverse colon. The pancreas was uniformly brown and had fairly distinct lobular markings. Lymph nodes along the bile duct and head of the pancreas were brown and enlarged up to 3 to 4 cm. in size. The gastric mucosa had a slightly brownish tinge, and the adrenal glands were browner than normal. The cortex of the left tibia, femur, and ribs and the tables of the skull were thin and crushed easily. The bone marrow was abundant and red-pink. The skin was somewhat darker than normal and slightly icteric. The heart weighed 400 gm., being otherwise entirely normal.

Histologically, throughout the liver the architecture was greatly distorted by irregular broad bands of fibrous tissue often linking the portal tracts one with another and, less frequently, the portal tracts with the hepatic veins. Within these fibrous bands there was a moderate degree of proliferation of the smaller bile ducts, and considerable numbers of lymphocytes were found in some areas. Hemosiderosis was quite diffuse, the greater amounts of pigment being present in the hepatic cord cells, Kupffer cells, and in macrophages in the fibrous bands. In the cytoplasm of the hepatic cord cells, the pigment most frequently was centrally placed, being concentrated around the nucleus. A moderate amount of pigment was found in the connective tissue fibers in the fibrous bands and in the epithelial cells of some bile ducts. There was a coarse interlobular fibrosis of the pancreas, and in many areas a focal, fine, intralobular fibrosis with isolation of the pancreatic acini. Iron pigment was found in extremely large amounts in the acinar cells, in lesser amounts in some of the cells of the islets of Langerhans, and in small amounts in macrophages and connective tissue cells and in the epithelium of some ducts.

The sinuses of the lymph nodes contained numerous macrophages filled with hemosiderin. The mucosal cells of the gastric glands contained large amounts of iron pigment, present almost exclusively on the secretory side of the cell. Iron pigment was present also in the zona glomerulosa of the adrenal glands, the cells lining distal convoluted tubules of the kidney, the glandular cells of parathyroid glands, the lining cells of the thyroid follicles, the glandular cells of the anterior lobe of the pituitary body, and in histiocytes of the lung and bone marrow. Other significant findings consisted of pericardial effusion (200 cc.), and right pleural effusion (150 cc.).

Case 5

Extensive hepatic and pancreatic fibrosis and siderosis in a 6-year-old Italian male who received large amounts of oral iron for prolonged intervals of time (275 mg. per day for 5½ years) and 42 gm. of iron by multiple transfusions.

The patient, an Italian male, was first seen at the New York Hospital on December 31, 1934, at the age of 4½ years. He had been well until the age of 7 months when pallor and severe anemia were noted. From 7 months until 4½ years of age treatment consisted of oral iron, ventriculin, liver extract, raw liver, and transfusions every 4 to 8 weeks in an effort to maintain the hemoglobin level. Examination revealed pallor, marked enlargement of the liver and spleen resulting in abdominal distention, a large head with puffiness of the face, a systolic heart murmur, and enlarged cervical and inguinal lymph nodes. The blood count showed: hemoglobin, 4.4 gm. per 100 cc.; red blood cells, 2 millions per cmm.; platelets, 120,000 per cmm.; white count, 11,000 per cmm. with a differential count of 23 per cent polymorphonuclear leukocytes, 3.5 per cent metamyelocytes, 3 per cent premyelocytes, 1 per cent basophilic myelocytes, 2 per cent eosinophils, 63.5 per cent lymphocytes, 3 per cent normoblasts, 0.5 per cent blast forms, and 0.5 per cent reticulocytes. The red blood cells showed anisocytosis, poikilocytosis, microcytosis, and basophilic stippling. The urine was negative for urobilinogen. Icterus index varied from 11 to 15 units. A red cell fragility test showed that hemolysis began at 0.36, and was complete at 0.26 (control, 0.44 and 0.34). Gastric analysis showed no free hydrochloric acid after a test meal of alcohol; however, a 30-minute gastric sample after a histamine test contained a total hydrochloric acid of 20 degrees. There was no retention of bromsulfalein after ½ hour. Roentgenograms of the long bones revealed thinning of the cortices and the shafts. The patient received 12 transfusions without improvement and splenectomy was performed in February, 1935. Thereafter until death in February, 1937, the patient received 2.7 gm. of iron ammonium citrate daily and 6 capsules of lextron daily, and was admitted to the hospital nine times for blood transfusions because of severe anemia (usually about 5 gm. of hemoglobin per 100 cc.). In October, 1936, edema of the legs was noted. Chemical examination of the blood at that time revealed cholesterol of 215 mg. per cent; urea nitrogen, 11 mg. per cent; total protein, 7.3 gm. per cent; and icterus index, 75. In February, 1937, he was admitted because of anemia and edema of the ankles and face. His heart was enlarged as before and there was massive enlargement of the liver. The urine contained 2 plus albumin; urea nitrogen was 10 mg. per cent; total protein, 7.2 gm.; albumin, 2.5 gm.; globulin, 4.71 gm. per cent; icterus index, 66; and total cholesterol, 205 mg. per cent. Edema increased and death occurred 1 week later.

Necropsy was performed 2 hours post mortem. The liver was markedly enlarged, weighing 2060 gm. (normal, 680 gm.*), and was firm and deep brown. The hepatic lobules were conspicuous and the portal areas depressed. The central portion of each lobule was yellow over a 2 to 3 mm. area, giving the cut surface a speckled appearance. The pancreas was deep reddish brown and slightly firmer than normal. Lymph nodes were enlarged in all sites and brown. The heart weighed 220 gm. (normal, 100 gm.), the left ventricle measuring 10 mm. and the right 4 mm. in thickness. The myocardium was reddish brown. The mucosa of the stomach was faintly tinged with brown and Peyer's

* Normal weights used throughout this paper are taken from Coppolletta and Wolbach.[20]

patches were enlarged and brownish pink. The bones were normal except for a brown discoloration of the marrow. The skin was a brownish yellow-green and the sclera light green.

Irregular fibrous bands radiating from the portal tracts divided the parenchyma into nodules of varying size. Within the fibrous tissue there was minimal bile duct proliferation, if any, and only a scanty infiltration with lymphocytes. Extremely large amounts of hemosiderin pigment were present diffusely in the hepatic cord cells throughout the liver lobules. The pigment was usually concentrated around the nucleus; however, at times the nucleus was displaced to the periphery of the cell by the central mass of pigment. In hepatic cells with lesser degrees of pigmentation, the hemosiderin was principally found in the cytoplasm between the nucleus and the bile capillary. Lesser quantities of hemosiderin were found in the Kupffer cells, macrophages, and connective tissue cells of the fibrous bands, and in the epithelial cells of some of the smaller bile ducts. In Sudan IV preparations lipids were demonstrated in vacuoles of various sizes within the cytoplasm of the hepatic cells of the inner half of the lobule. There was a diffuse fine interlobular and intralobular fibrosis of the pancreas and in addition occasional focal areas of more extensive fibrosis were seen. A moderate number of lymphocytes and occasional neutrophils were present diffusely in the stroma. The pancreatic ducts were entirely normal and the islets of Langerhans devoid of degenerative changes or fibrosis. Large amounts of hemosiderin were present in the acinar cells and the cells of the islets of Langerhans, and lesser amounts were found in the connective tissue cells and macrophages.

Iron pigment was present in large amounts in the cytoplasm of macrophages in the sinuses of lymph nodes, and around the nuclei of the myocardial fibers. It was found likewise in the cells lining the glands of the stomach, especially along the inner margin of the cytoplasm. Large numbers of macrophages were filled with iron pigment in the villi of the ileum, but iron pigment was not observed within the glandular cells.

Other findings consisted of generalized subcutaneous edema, peritoneal effusion (150 cc.), and pericardial effusion (50 cc.).

Case 8

Extensive visceral siderosis with fibrosis of the liver and pancreas in a 7-year-old Italian male who had received multiple blood transfusions periodically since the age of 9 months (total, 16 gm. of iron).

The patient, an Italian male, developed intermittent vomiting, pallor, and hepatosplenomegaly at the age of 9 months. The hemoglobin was 8.2 gm. per 100 cc. and

red blood cells were 3.2 millions per cmm. Anisocytosis, poikilocytosis, microcytosis, and large numbers of normoblasts were noted in smears. From 1943 to 1945 seven hospitalizations were required for transfusions, and subsequently transfusions were given every 2 to 4 weeks for the next 3½ years. This regimen maintained hemoglobin levels in the general range of 7 to 10 gm. per 100 cc. After 1948 it became progressively difficult to maintain these hemoglobin levels despite greater frequency of transfusions, and during this period the spleen had increased greatly in size and extended into the pelvis. Splenectomy was performed in April, 1949, and thereafter there was pronounced clinical improvement and higher hemoglobin levels were maintained than preoperatively despite less frequent administration of blood. In August, 1950, he developed a sudden illness characterized by high fever, convulsions, and coma, and died 2 days later.

Necropsy * was performed 2 hours post mortem. The liver was markedly enlarged, weighing 2069 gm. (normal, 680 gm.) and was adherent by fibrous adhesions to the anterior surface of the diaphragm. The surface was smooth and on section the parenchyma was homogeneously brown and firm. The pancreas likewise was diffusely brown, firm, and weighed 110 gm. Lymph nodes around the bile ducts ranged to 2.5 cm. in size and were diffusely brown as were the hilar, periaortic, and mesenteric lymph nodes. The heart weighed 185 gm. (normal 100 gm.) and was dark red and firm. The left ventricle measured 1 cm. and the right ventricle 0.3 cm. in thickness, respectively. The bodies of the vertebrae were friable, easily cut, and the marrow red-brown and hyperplastic. The tables of the skull were thin and the marrow abundant. The skin was unusually dark and dusky.

The normal lobular architecture of the liver was completely distorted by bands of fibrous tissue which linked portal areas with one another and with hepatic veins (Fig. 5). Within the fibrous bands bile duct proliferation was minimal and only scattered chronic inflammatory cells were present. Hemosiderosis was diffuse throughout the liver lobule. Large amounts of iron pigment were present in the liver cord cells, Kupffer cells, and macrophages in the portal areas, and lesser amounts were found in the epithelial cells of some of the smaller bile ducts, and connective tissue cells (Fig. 6). Within the cytoplasm of the hepatic cells the pigment was often concentrated around the nucleus or between the nucleus and the bile capillary, but in some cells it was distributed diffusely. The pancreatic alterations comprised a diffuse fine interlobular and intralobular fibrosis, and in addition there were focal areas of more extensive fibrosis in which fibrous tissue completely isolated the pancreatic acini (Fig. 7). Hemosiderosis was diffuse throughout the pancreas and the pigment was found most

* By the Pathology Staff of the St. Joseph's Hospital, Stamford, Conn., who kindly provided the gross description and blocks for histologic study.

abundantly in the acinar cells. Within the cytoplasm of the acinar cells the pigment was distributed diffusely or more commonly concentrated between the nucleus and the alveolar lumen (Fig. 8). Varying degrees of hemosiderin deposition were found in all of the islets of Langerhans, the pigmentation being heavier in some cells than others. Lesser degrees of pigmentation were present in macrophages and connective tissue cells of the interstitium and in the epithelial cells of some of the smaller pancreatic ducts. Degenerative changes or fibrosis of the islets was not present.

Extremely large amounts of hemosiderin were present in macrophages in the sinuses of lymph nodes, and small amounts in similar cells in the thymus and lung. A large amount of hemosiderin was found in the myocardial fibers, usually concentrated around the nucleus. Fibrosis of the heart was absent. A small amount of iron pigment was found in the cells of the adrenal zona glomerulosa. No abnormalities of the brain were present either grossly or microscopically.

Comment

The visceral siderosis and fibrosis in the cases here reported are essentially the same as those seen in other conditions, notably in idiopathic hemochromatosis,[10,11] in exceptional cases of transfusion hemosiderosis with cirrhosis,[12-17] in a small proportion of cases of nutritional cytosiderosis,[18,19] and in rare cases of chronic anemia in which the patients were given large doses of oral iron over prolonged periods.[21] Even so, the pathogenesis of the siderosis and the fibrosis, and their relationships to one another, remain largely undefined. For, although it is often assumed that excessive deposits of iron pigment may lead to visceral fibrosis, actually it is not known that iron pigment is injurious to human hepatic and pancreatic tissues. Indeed, in many cases of transfusion hemosiderosis and nutritional cytosiderosis iron deposition is excessive in the liver and other viscera while fibrosis is absent,[12-19] and much the same has been true in some cases of idiopathic hemochromatosis.[10,11] Furthermore, the attempts made thus far to induce visceral fibrosis in several species of experimental animals rendered hemosiderotic by various means have been unsuccessful.[9,22-28] The findings as a whole make it seem probable that some factor or factors other than the iron deposits *per se* are concerned in the pathogenesis of the visceral fibrosis. In this relation it may be noted that severe chronic anemia has been a common factor in the cases of so-called transfusion hemosiderosis with cirrhosis of the liver,[12-17] in a case of hemochromatosis following the administration of oral iron over

a 12-year period,[21] and in the cases of Cooley's anemia with fibrosis, as reported here and elsewhere.[7,8] This factor may well be concerned in the pathogenesis of the siderosis in Cooley's anemia, for Dubach, Callender, and Moore[29] have shown that patients with non-iron-deficient anemias may absorb more iron from the gastro-intestinal tract than is utilized in the synthesis of hemoglobin; but whether it is also implicated in the pathogenesis of the visceral fibrosis of Cooley's anemia cannot be decided on the evidence at hand.

Summary

Thirteen cases of Cooley's anemia that had been under clinical observation for periods of 2 to 27 years were studied anatomically, 6 at post-mortem examination and 7 others by means of biopsies. Microscopic examinations disclosed widespread visceral siderosis together with fibrosis of the liver and pancreas in 4 of the 6 cases examined post mortem, while both siderosis and fibrosis were found in the liver specimens procured from the 7 additional cases. In 3 instances the widespread cirrhosis of the liver and fibrosis of the pancreas, together with visceral siderosis including involvement of hepatic cord cells and epithelium of some of the bile ducts, acinar and islet cells of the pancreas, and connective tissue fibers of the fibrous bands in liver and pancreas, comprised anatomical changes identical with those that characterize idiopathic hemochromatosis, while in 8 additional cases the changes were similar though less extensive. Diabetes mellitus was present in one of the cases with extensive pancreatic fibrosis.

Histologic findings and chemical analyses indicated that the amount of iron present in the liver in several cases exceeded the amount given to the respective patients by repeated transfusions. These findings are similar to those previously noted by others in cases of visceral siderosis following repeated transfusions, and they made it seem likely that an excessive absorption of iron from the gastro-intestinal tract contributed significantly to the siderosis.

Although siderosis and fibrosis generally paralleled one another in the various cases, definitive evidence of a causal relationship between the two was lacking. Furthermore, the fibrosis could not be closely correlated with the duration of the disease process, and the findings make it seem likely that some factor or factors other than the duration of the disease *per se* must have been involved in the pathogenesis of the hepatic and pancreatic fibrosis. The fact was noted that chronic anemia of long duration was a common factor in the cases of Cooley's anemia with fibrosis and in certain other cases of so-called

transfusion hemochromatosis, but whether this factor was responsible for the fibrosis could not be decided. In essence the cause of the fibrosis remains undisclosed.

Acknowledgment is given to Mr. Julius Mesiar for preparation of the photomicrographs.

REFERENCES

1. Whipple, G. H., and Bradford, W. L. Mediterranean disease—thalassemia (erythroblastic anemia of Cooley). *J. Pediat.*, 1936, **9**, 279–311.
2. Whipple, G. H., and Bradford, W. L. Racial or familial anemia of children associated with fundamental disturbances of bone and pigment metabolism (Cooley-von Jaksch). *Am. J. Dis. Child.*, 1932, **44**, 336–365.
3. Cooley, T. B., Witwer, E. R., and Lee, P. Anemia in children. With splenomegaly and peculiar changes in the bones: Report of cases. *Am. J. Dis. Child.*, 1927, **34**, 347–363.
4. Yaguda, A. Erythroblastosis in the newborn and in early childhood. *Am. J. Clin. Path.*, 1935, **5**, 266–298.
5. Koch, L. A., and Shapiro, B. Erythroblastic anemia. Review of cases reported showing roentgenographic changes in bones and five additional cases. *Am. J. Dis. Child.*, 1932, **44**, 318–335.
6. Baty, J. M., Blackfan, K. D., and Diamond, L. K. Blood studies in infants and in children. I. Erythroblastic anemia; a clinical and pathologic study. *Am. J. Dis. Child.*, 1932, **43**, 667–704.
7. Frumin, A. M., Waldman, S., and Morris, P. Exogenous hemochromatosis in Mediterranean anemia. *Pediatrics*, 1952, **9**, 290–294.
8. Howell, J., and Wyatt, J. P. Development of pigmentary cirrhosis in Cooley's anemia. *A. M. A. Arch. Path.*, 1953, **55**, 423–431.
9. Finch, C. A., Hegsted, D. M., Kinney, T. D., Thomas, E. D., Rath, C. E., Haskins, D., Finch, S., and Fluharty, R. G. Iron metabolism: the pathophysiology of iron storage. *Blood*, 1950, **5**, 983–1008.
10. Sheldon, J. H. Haemochromatosis. Oxford University Press, 1935, 382 pp.
11. Sheldon, J. H. Haemochromatosis. *Lancet*, 1934, **2**, 1031–1036.
12. Kark, R. M. Two cases of aplastic anaemia, one with secondary haemochromatosis following 290 transfusions in nine years, the other with secondary carcinoma of the stomach. *Guy's Hosp. Rep.*, 1937, **87**, 343–353.
13. Zeltmacher, K., and Bevans, M. Aplastic anemia and its association with hemochromatosis. *Arch. Int. Med.*, 1945, **75**, 395–403.
14. Schwartz, S. O., and Blumenthal, S. A. Exogenous hemochromatosis resulting from blood transfusions. *Blood*, 1948, **3**, 617–640.
15. Muirhead, E. E., Crass, G., Jones, F., and Hill, J. M. Iron overload (hemosiderosis) aggravated by blood transfusions. *Arch. Int. Med.*, 1949, **83**, 477–501.
16. Wyatt, J. P., Mighton, H. K., and Moragues, V. Transfusional siderosis. *Am. J. Path.*, 1950, **26**, 883–897.
17. Graef, I., Gordon, B. S., Newman, W., Olivetti, R. G., and Klein, B. Observations in exogenous hemochromatosis apparently due to multiple transfusions. (Abstract.) *Am. J. Path.*, 1952, **28**, 538–539.

18. Gillman, J., and Gillman, T. Structure of the liver in pellagra. *Arch. Path.*, 1945, 40, 239–263.

19. Gillman, J., Mandelstam, J., and Gillman, T. A comparison of chemical and histological estimations of the iron and copper content of the livers of Africans in relation to the pathogenesis of cytosiderosis and cirrhosis (haemochromatosis). *South African J. M. Sc.*, 1945, 10, 109–136.

20. Coppolletta, J. M., and Wolbach, S. B. Body length and organ weights of infants and children. A study of the body length and normal weights of the more important vital organs of the body between birth and twelve years of age. *Am. J. Path.*, 1933, 9, 55–70.

21. Wallerstein, R. O., and Robbins, S. L. Hemochromatosis after prolonged oral iron therapy in a patient with chronic hemolytic anemia. *Am. J. Med.*, 1953, 14, 256–260.

22. Rous, P., and Oliver, J. Experimental hemochromatosis. *J. Exper. Med.*, 1918, 28, 629–644.

23. Cappell, D. F. The late results of intravenous injection of colloidal iron. *J. Path. & Bact.*, 1930, 33, 175–196.

24. Polson, C. J. Failure of prolonged administration of iron to cause haemochromatosis. *Brit. J. Exper. Path.*, 1933, 14, 73–76.

25. Kinney, T. D., Hegsted, D. M., and Finch, C. A. The influence of diet on iron absorption. I. The pathology of iron excess. *J. Exper. Med.*, 1949, 90, 137–146.

26. Hegsted, D. M., Finch, C. A., and Kinney, T. D. The influence of diet on iron absorption. II. The interrelation of iron and phosphorus. *J. Exper. Med.*, 1949, 90, 147–156.

27. Brown, E. B., Moore, C. V., Reynafarje, C., and Smith, D. E. Intravenously administered saccharated iron oxide in the treatment of hypochromic anemia. Therapeutic results, potential dangers and indications. *J. A. M. A.*, 1950, 144, 1084–1089.

28. Wyatt, J. P., and Howell, J. Experimental induction of iron overload in the rat. I. Morphological alterations due to dietary siderosis. *A. M. A. Arch. Path.*, 1953, 55, 466–474.

29. Dubach, R., Callender, S. T. E., and Moore, C. V. Studies in iron transportation and metabolism. VI. Absorption of radioactive iron in patients with fever and anemias of various etiology. *Blood*, 1948, 3, 526–540.

[*Illustrations follow*]

LEGENDS FOR FIGURES

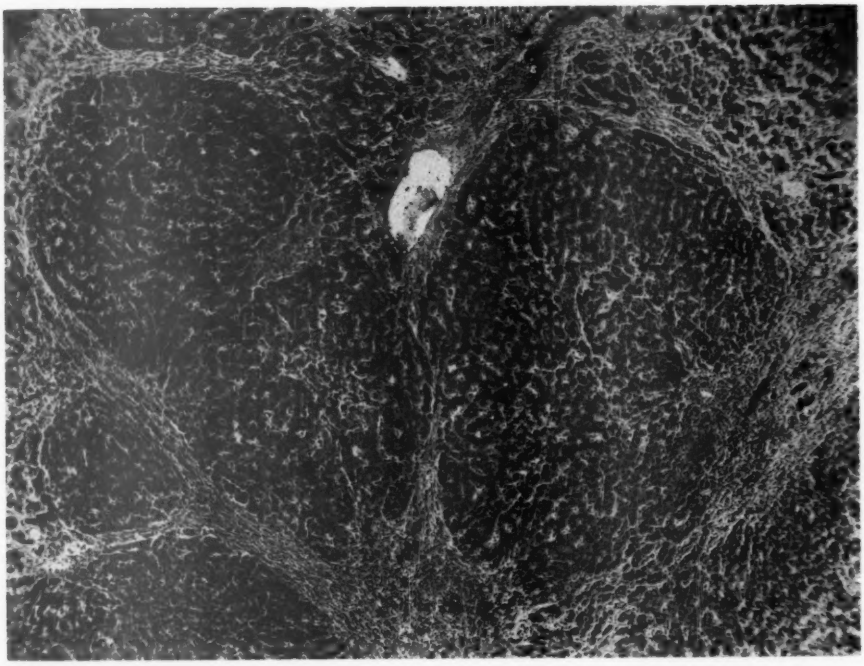
FIG. 1. Case 1. Grade 4 fibrosis of liver. Broad bands of fibrous tissue link portal areas with one another and with hepatic veins forming parenchymal nodules of various sizes. Masson's trichrome stain. × 50.

FIG. 2. Case 1. Grade 4 siderosis of liver. Diffuse and extensive hemosiderin pigmentation of connective tissue fibers, macrophages, cells forming the walls of blood vessels and bile ducts all within a fibrous band, as well as of the hepatic cord cells. Gomori's ferrocyanide stain, hematoxylin counterstain. × 90.

1

2

1



2

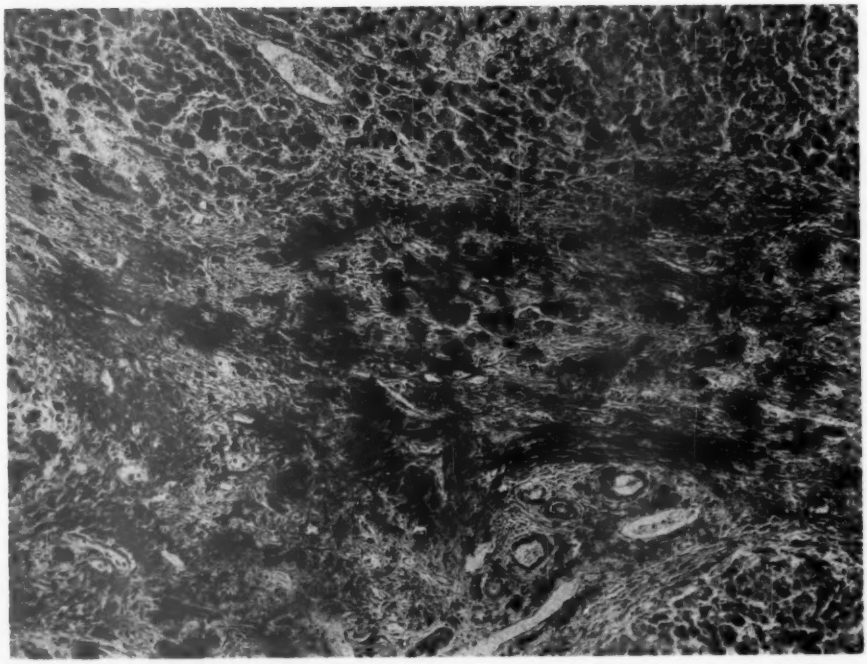
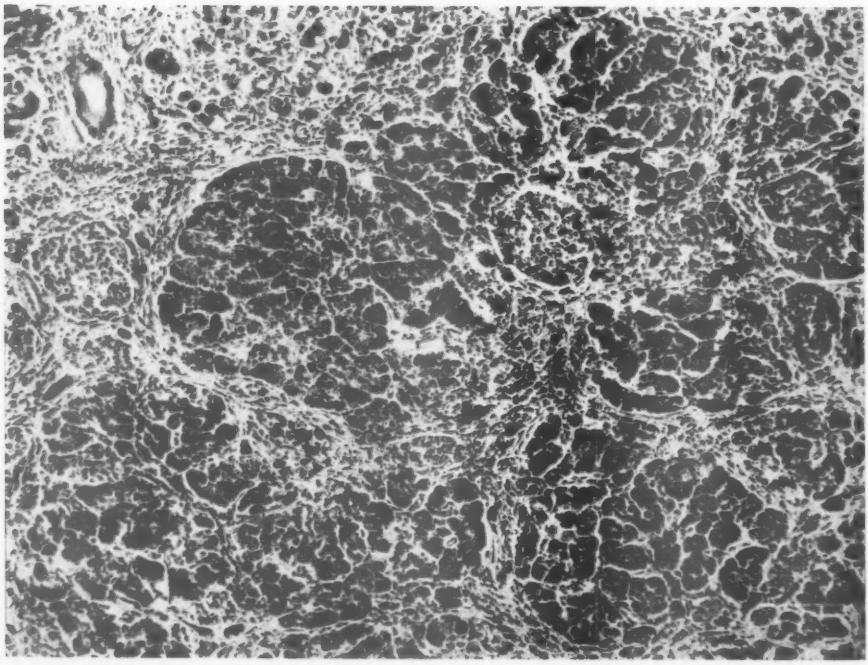
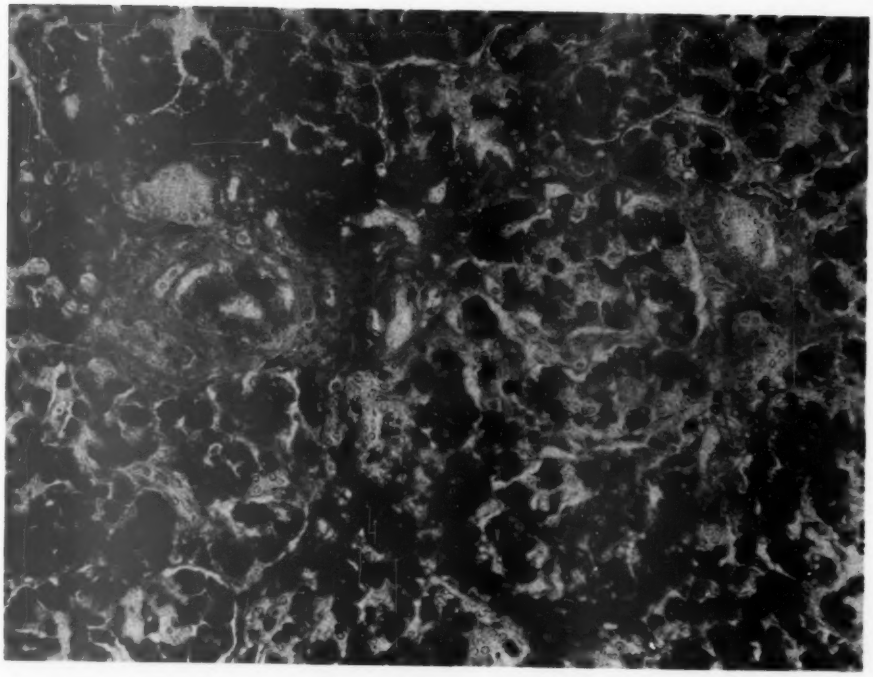


FIG. 3. Case 1. Grade 4 fibrosis of pancreas. Diffuse interlobular and intralobular fibrosis of pancreas. Coarse bands of fibrous tissue divide the parenchyma into nodules of various sizes. Masson's trichrome stain. × 90.

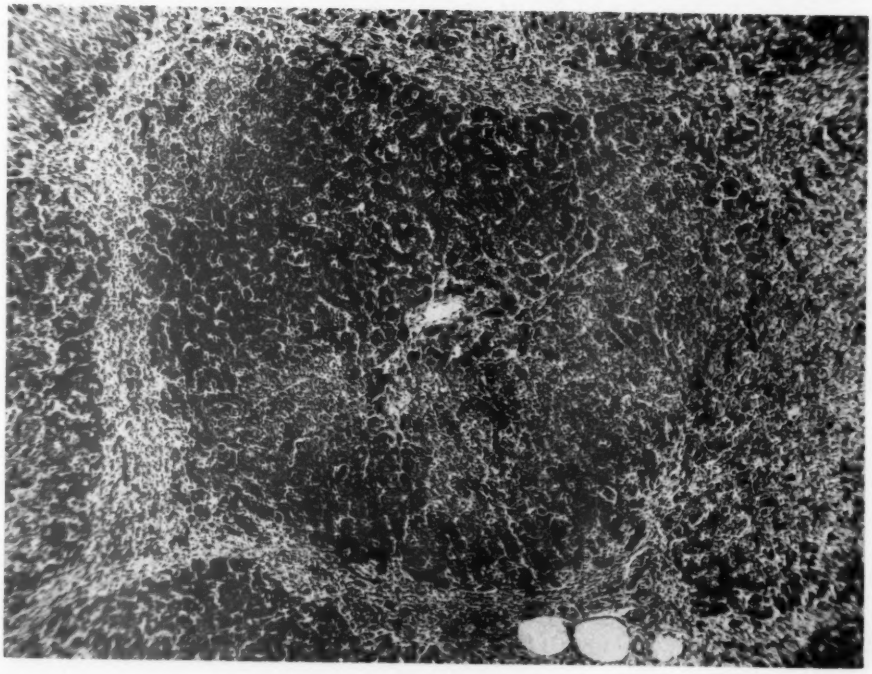
FIG. 4. Case 1. Grade 4 siderosis of pancreas. Diffuse extensive hemosiderin deposits in acinar epithelium, islet of Langerhans, epithelial cells of one of the pancreatic ducts, and some of the connective tissue cells. Gomori's ferrocyanide stain, hematoxylin counterstain. Wratten F filter. × 180.

FIG. 5. Case 8. Grade 4 fibrosis of liver. Complete encirclement of a liver lobule by bands of fibrous tissue which connect the portal areas one with another. Masson's trichrome stain. × 50.





4

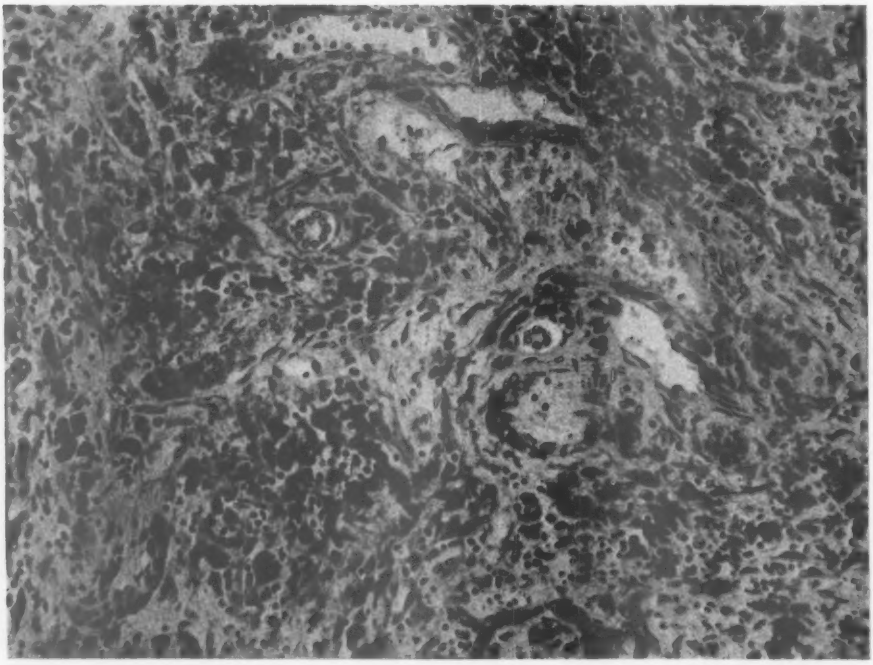


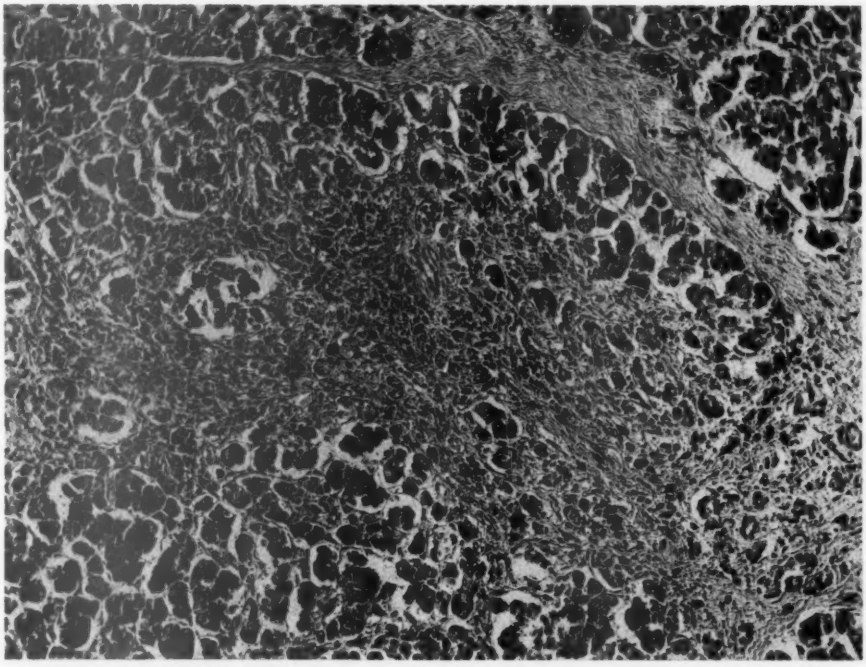
5

FIG. 6. Case 8. Grade 3 siderosis of liver. Extensive hemosiderin deposits in the connective tissue cells, macrophages, epithelial cells of some of the bile ducts, and cells forming the walls of some of the blood vessels. Gomori's ferrocyanide stain, hematoxylin counterstain. × 180.

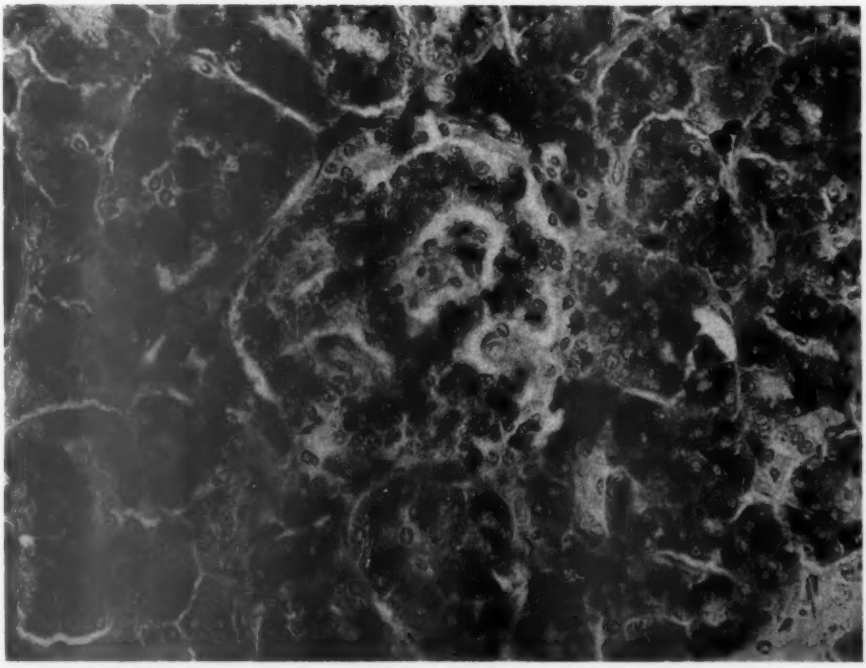
FIG. 7. Case 8. Grade 2 fibrosis of pancreas. One of the focal areas of intralobular fibrosis in which fibrous tissue extends between and around the pancreatic acini. Masson's trichrome stain. × 90.

FIG. 8. Case 8. Grade 3 siderosis of pancreas. Extensive hemosiderin deposits in the cells of the acini and islet of Langerhans, macrophages in the interstitium, and in a few of the connective tissue fibers. Gomori's ferrocyanide stain, hematoxylin counterstain. Wratten F filter. × 280.





7



8

QUANTITATIVE STUDIES OF ACUTE NEPHROTOXIC NEPHRITIS IN RATS*

MARVIN S. WEINREB, M.D., KATHRYN H. SOULES, A.B., and ROBERT W. WISSLER, M.D.

(From the Department of Pathology, the University of Chicago, Chicago 37, Ill.)

The effects of the active immunization of one species with the kidney tissue of another species have been investigated for over 50 years[1,2] but the fundamental processes by which the resulting nephrotoxic antisera produce renal lesions and profound proteinuria are still obscure. Even the histopathologic nature of the renal lesions resulting from the injection of anti-rat-kidney rabbit serum into rats has been described quite differently by various observers. Thus, Masugi[3] emphasized the inflammatory and proliferative changes in the endothelial elements of the glomerular tuft with resulting tuft synechiae, while Smadel[4] interpreted the essential glomerular lesions as primarily degenerative with the major changes occurring in the capillary basement membrane with little, if any, proliferative or inflammatory reaction. The results of the more recent investigations have been similarly divergent.[5-9] Some of these variations in interpretation may have resulted from differences in technique of immunization, but it seems more likely that the major differences have resulted from variations in the quantity of antisera employed and the difficulties in interpreting the glomerular changes with the staining procedures used.

This study was undertaken in an attempt to define the essential nature of the glomerular changes resulting from the intravenous injection of varying quantities of anti-rat-kidney serum into rats, using some of the newer histologic techniques which have proved of value in studying human renal disease. An attempt has been made to correlate the observed histologic changes with the degree of proteinuria which each animal demonstrated. Since it has become apparent that the injection of nephrotoxic antiserum in the rat results in a state closely simulating that seen clinically in human nephrotic nephritis and lipid nephrosis, the results may prove helpful in understanding the essential defect in these obscure diseases.

MATERIALS AND METHODS

Preparation of Antigen and Antiserum. About 250 ml. of lyophilized rabbit anti-rat-kidney serum and a smaller amount of normal rabbit

* Supported in part by American Cancer Society Institutional Grant No. 14E, to the University of Chicago.

Received for publication, July 10, 1953.

serum were prepared as follows: Rat kidneys, perfused with cold Ringer's solution, were removed sterilely and frozen quickly. They were then dried to constant weight at −20°C., in a vacuum desiccator over P_2O_5. A fine powder was prepared from these dried kidneys by grinding them in a sterile mortar and pestle and the powder was stored at −20°C., in a vacuum over P_2O_5. The serum used was pooled from three groups of rabbits injected 3 days a week over a 12-week period with 1 week of rest in each 4. The rabbits were bled during the 12th, 13th, and 14th weeks and the sterile serum was lyophilized and stored in a vacuum desiccator at −20°C. Two groups of rabbits were injected with kidney which was autolyzed at 4°C. for 48 hours, as described by Smadel.[4] The other group received antigen prepared by the method of Hektoen and Welker,[10] being autolyzed for 48 hours at room temperature after mixing with aluminum hydroxide. All rabbits received daily doses equivalent to 5 ml. of a 5 per cent whole kidney suspension. One group of 5 rabbits received the Smadel antigen intraperitoneally, another group received the antigen containing aluminum hydroxide intramuscularly, and the third group received the Smadel antigen as follows: 1 ml. intraperitoneally, 1 ml. intramuscularly, 1 ml. subcutaneously, 1 ml. intravenously, and five intradermal injections of 0.2 ml. each.

The sera were reconstituted with sterile distilled water shortly before use. Complement fixation tests showed all the sera to have strong anti-kidney titers and very low concentrations of antibodies to rat erythrocytes and rat serum.

Injection of Anti-Kidney Serum into Rats and Collection of Urine. The animals were injected slowly with anti-kidney serum in a tail vein under light anesthesia.

Urine was collected by placing single animals in metabolism cages for periods of time ranging from 8 to 18 hours. While urine was collected they were given water but no food. Urine ran down paraffin-coated funnels into small bottles containing 2 to 4 ml. of toluene.

Urinalysis. The urine volume was measured and the urine was filtered. Protein was measured quantitatively by the method of Kingsbury, Clark, Williams, and Post.[11] Unconcentrated samples were used for microscopic examination.

Animals and Care. Male Sprague-Dawley rats were used exclusively, their weight varying with the experiment. Purina laboratory chow and water *ad libitum* were given the animals unless otherwise noted.

Histologic Procedures. The rats were sacrificed under ether anesthesia by opening the thorax and cutting the aorta and vena cava.

They were necropsied immediately and samples of kidneys, lungs, and liver were taken from all animals; in some animals the heart, adrenal glands, and spleen also were obtained. Buffered formalin, Zenker's fluid, and cold acetone were the fixatives employed and the sections were stained with hematoxylin and eosin, Mallory's azan,[12] Hotchkiss' periodic acid-Schiff's reaction,[13] toluidine blue; and Rinehart and Abul-Haj's modifications[14] of the Hale stain.[15] Of the latter two, one combines colloidal iron and cochineal; the other, colloidal iron and the periodic acid-Schiff reaction.

Experimental

Three experiments were performed and will be described separately.

Experiment 1

Plan. The first experiment was planned to study the effect of dosage on the severity of the nephritis produced. Twelve rats weighing about 200 gm. were divided into six pairs. Five pairs were given single injections of 0.5, 1.0, 1.5, 2.0, and 3.0 ml. of anti-kidney serum intravenously. The control pair was given normal rabbit serum, one rat receiving 0.5, the other 3.0 ml. One animal of each pair was immediately put into a metabolism cage. The first urine specimens represented the output of the first 8 hours after injection. Subsequently, the urine specimens were collected daily for periods of between 8 and 18 hours, the 2 animals receiving the same dose being alternated in the metabolism cages. The animals were sacrificed at 5 days.

Results. Control urine examinations of all rats showed protein values of 13.8 mg. per 24 hours or less, most values being below 5 mg. per 24 hours. Casts were absent. Three hours after injection one rat which had received 3.0 ml. of anti-kidney serum was found dead. It had hydrothorax, pulmonary edema, ascites, and dark red swollen kidneys. All of the others survived.

At 8 hours there was moderate polyuria and elevated proteinuria in all animals except the controls (Table I). Proteinuria increased, reaching a peak at 2 days. It then declined in one of the lowest dosage rats while the other showed no rise. Proteinuria remained high in the other pairs of rats. Granular and hyaline casts were seen first on the day after injection and they continued to be observed through the experiment in all animals, including controls. Only rarely were red and white cells seen. No doubly refractile fat was found. The highest urine protein value recorded was 300 mg. per 24 hours. Injections up to 2.0 ml. resulted in increased proteinuria, but 3.0 ml. was no more effective than 2.0 ml. (Table I). No rats showed clinical ascites or

edema. At necropsy the rats receiving low dosages and the controls had grossly normal kidneys; those on higher dosages showed enlarged, pale, flabby kidneys. All had enlarged spleens except the controls.

Microscopically, no fatty lesions were seen in the kidneys. The

TABLE I

Urine Protein Values in Rats of Experiment 1

Anti-kidney serum given	Day of determination of urine protein	Animal no.	Urine protein		Animal no.	Urine protein	
ml.			*gm. %*	*mg./ 24 hrs.*		*gm. %*	*mg./ 24 hrs.*
0.5		1			2		
	−1		0.10	3.0		0.01	1.0
	0*		0.09	3.6		0.01	0.7
	0†		0.15	5.6			
	2		0.30	13.5		0.04	2.2
	4		0.20	7.5		0.02	1.5
	5					0.02	1.0
1.0		3			4		
	−1		0.03	1.8		0.02	2.0
	0*		0.04	1.2		0.04	2.4
	0†		0.50	67.5			
	2		1.50	60.0		1.00	55.0
	4		1.50	90.0		0.50	43.5
	5					0.50	45.0
1.5		5			6		
	−1		0.01	1.4		0.025	2.8
	0*		0.04	2.4		0.06	3.6
	0†		1.00	165.0			
	2		2.50	150.0		2.50	162.0
	4		1.50	146.0		1.00	160.0
	5					2.50	300.0
2.0		7			8		
	−1		0.10	2.6		0.04	2.4
	0†		1.20	72.0			
	2		4.00	180.0		3.00	135.0
	4		2.50	225.0		1.25	109.0
	5					1.00	240.0
3.0		9			10		
	−1		0.18	3.6		0.12	13.2
	0†		2.00	75.0		Died	
	2		5.00	125.0			
	4		5.00	75.0			
0.0‡		11			12		
	−1		0.04	4.4		0.15	5.2
	0†		0.10	6.0			
	2		0.10	6.0		0.03	1.5
	4		0.10	6.0		0.10	10.7
	5					0.10	6.7

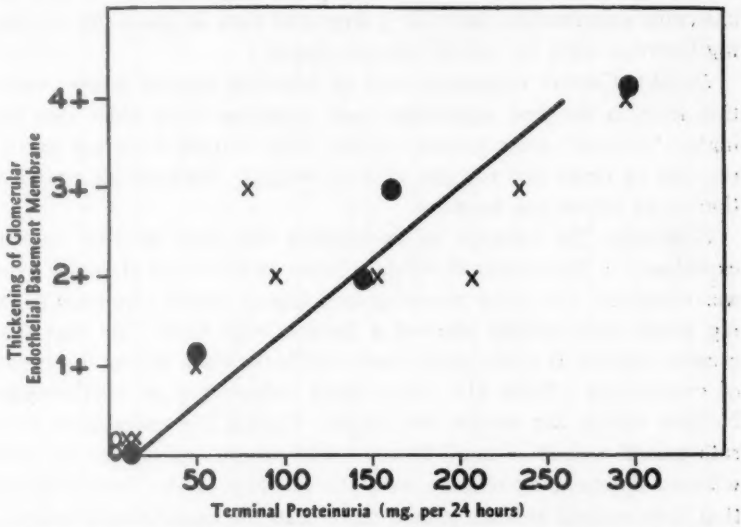
* Urines obtained 3½ hours before injection.

† Urines obtained 8 hours after injection.

‡ Rat 11 received 0.5 cc. and rat 12 received 3.0 cc. of normal rabbit serum.

more conspicuous changes were a thickening of the glomerular capillary basement membrane and a decrease in the size of the capillary lumina of the tuft. This was observed best in sections prepared by the periodic acid-Schiff reaction and was proportional to the severity of the proteinuria. It also was noted that the capillary loops were sepa-

rated in the experimental animals as compared to the controls. The spaces between the loops were occupied by mucoid material which did not stain with hematoxylin and eosin, periodic acid-Schiff's, or Mallory's azan. This mucoid material, as well as the thickness of the basement membrane, was increased in all rats with moderate and marked proteinuria. These increases were correlated with the rise in protein output (Text-fig. 1). For example, rats 5, 6, 7, and 8 had more



Text-fig. 1. The correlation between the urinary protein excretion at the last determination before sacrifice and the estimated thickening of the glomerular endothelial basement membrane. Experiment 1. Cross indicates anti-kidney serum animals; open circle indicates control animals; solid circle indicates mean points.

proteinuria, thicker basement membranes, and more mucoid material than the other rats. This change, as well as the others mentioned, was diffuse and involved most of the glomeruli of both kidneys in animals affected. Proliferative changes were absent in the glomeruli and there were no tubular lesions. All other organs appeared normal. The rat which died after 3 hours showed a different picture. The glomerular capillaries contained many neutrophilic leukocytes, and occasional capillary loops were widely distended. Pulmonary edema and congestion also were seen.

Experiment 2

Plan. The second experiment was planned to study the lesions at various times after injection using a constant dose of anti-kidney serum. It also served to introduce the factor of animal age and size

since the rats used in this experiment were much older and heavier than those of the preceding experiment. Fourteen rats varying from 327 to 467 gm. were used.

The animals were divided into seven pairs. Twelve animals received a single 1.5 ml. intravenous injection of anti-kidney serum and 2 received 1.5 ml. of normal rabbit serum. The rats were sacrificed in pairs at 2, 4, 7, 10, 14, and 21 days after injection. Controls were sacrificed at 21 days. Urine was collected for protein determinations and microscopic examination daily for 5 days and then at gradually increasing intervals until the end of the experiment.

Results. Control urinalyses prior to injection showed greater variation than in the first experiment and generally these older rats had higher "normal" urine protein values. They ranged from 1.2 to 16.6 mg. per 24 hours per 100 gm. of body weight. Microscopic examination of all urines was negative.

Generally, the increase of proteinuria was less marked than in experiment 1, but occurred within 8 hours in all 6 rats of which urine was examined soon after receiving anti-kidney serum. Animals showing small rises usually showed a decline with time. The rats with greater degrees of proteinuria showed little decrease during the period of observation (Table II). Casts were present but no erythrocytes. Neither edema nor ascites was noted. During the experiment some rats gained weight in small amounts and others lost similar amounts with no apparent correlation with the severity of the disease except that both control animals gained more than the experimental animals. At sacrifice, the liver, kidneys, spleen, and adrenal glands were weighed. Expressed as per cent of body weight, the kidneys of all rats injected with anti-kidney serum and sacrificed after the second day following injection were heavier than those of the control rats, but the weight increases were not proportionate to the degree of proteinuria. Expressed in the same manner, all adrenal glands of experimental animals were heavier than those of the controls. Weights of spleen and liver did not change significantly. With the exception of a variable number of petechiae over the lung surfaces in 7 of 10 experimental rats sacrificed up to the 14th day, the other organs seemed grossly normal.

Examination of the kidneys microscopically showed the same changes seen in the previous experiment. Both the Hotchkiss and Mallory stains, which bring out the capillary basement membrane, showed that as early as 2 days after injection there was detectable

TABLE II

Urine Protein Values in Experiment 2 *

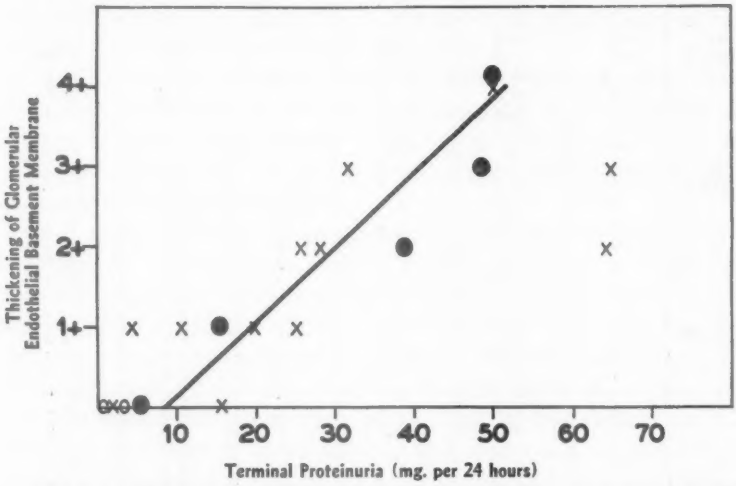
Animal no.	Day of determination of urine protein‡	Urine volume 24 hrs. (ml.)	Urine protein mg./24 hrs./ 100 gm.	Day of determination of urine protein	Urine volume 24 hrs. (ml.)	Urine protein mg./24 hrs./ 100 gm.
13	−2	8.4	3.4	−2	3.0	0.8
A and B	−1	5.0	3.0	−1	8.8	2.8
	0†	6.6	19.3	1	8.0	31.8
	1	5.4	19.8	2	22.8	15.1
	2	25.4	59.5			
14	−2	9.1	2.5	−2	5.6	7.4
A and B	−1	11.8	4.3	−1	8.6	11.9
	0†	16.8	25.7	1	3.6	29.8
	1	10.4	31.8	2	5.4	32.6
	2	9.4	14.4	3	6.0	24.8
	4	10.2	15.6			
15	−2	9.1	1.6	−2	4.0	2.4
A and B	−1	6.8	1.8	−1	7.2	7.0
	0†	19.5	6.5	1	16.6	100.0
	1	8.4	11.2	2	12.8	46.5
	2	5.6	7.4	3	9.4	57.0
	4	4.2	5.6	5	6.6	48.0
	7	3.4	4.6	7	14.8	67.0
16	−2	10.5	1.4	−2	8.6	2.7
A and B	−1	4.0	1.2	−1	10.4	4.3
	0†	36.0	11.0	1	6.6	25.4
	1	23.2	19.1	2	6.8	8.8
	2	8.0	9.7	3	5.4	11.8
	4	7.8	10.0	5	6.8	17.0
	7	2.0	3.8	7	5.6	7.0
17	−2	3.5	3.0	−2	3.4	1.8
A and B	−1	3.0	2.3	−1	4.4	4.8
	0†	4.8	24.6	1	6.8	22.0
	1	8.4	32.4	2	16.6	17.9
	2	5.2	20.0	3	11.4	32.0
	7	4.8	6.6	7	24.2	13.1
	14	6.8	43.5	14	10.2	25.0
18	−2	10.5	6.4	−2	4.6	4.5
A and B	−1	8.4	6.8	−1	4.8	4.9
	0†	5.1	27.4	1	9.2	37.6
	1	9.0	24.2	2	18.6	12.6
	2	9.4	12.6	3	14.0	19.0
	7	3.4	11.3	7	13.6	9.2
	14	6.2	11.0	14	13.8	19.8
	20	3.2	12.8	20	6.6	20.5
19	−1	16.0	4.2	−1	12.2	6.5
A and B	0†	15.6	4.7	1	11.0	4.2
	1	21.0	5.5	2	11.2	4.5
	2	20.0	5.2	3	9.4	2.4
	7	19.8	2.3	7	16.6	1.7
	14	23.6	2.8	14	24.2	4.3
	20	27.0	3.2	20	10.8	5.7

* All animals received 1.5 cc. of anti-kidney serum except 19A and 19B which received 1.5 cc. of normal rabbit serum.

† Urines taken 8 hours after injection.

‡ All animals sacrificed 24 to 48 hours after final urine protein determination except 16A and 16B which were sacrificed on day 11.

thickening of the basement membrane which encroached upon the capillary lumen in places. The mucoid material between capillary loops also was increased at 2 days and continued to increase in rats showing elevated proteinuria. The degree of thickening of the Hotchkiss-positive basement membrane as well as the amount of mucoid material was proportional to the degree of proteinuria of the animal at time of sacrifice (Text-fig. 2). For example, rats 14B, 17A, 17B,



Text-fig. 2. The correlation between the urinary protein excretion at the last determination before sacrifice and the estimated thickening of the glomerular endothelial basement membrane. Experiment 2. Cross indicates anti-kidney serum animals; open circle indicates control animals; solid circle indicates mean points.

and 18B, which, respectively, showed 24.8, 43.5, 25.0, and 20.5 mg. of urinary protein per 24 hours per 100 gm. just before sacrifice, all showed the above-mentioned changes to the greatest degree (Fig. 1). Rats 16A and 18A which showed early rises in urinary protein followed by decreases to values approaching those of controls, had normal appearing glomeruli. On comparing the controls in both experiments, it was seen that the older rats in the second experiment generally had thicker capillary basement membranes, which is notable in view of their greater "physiologic" proteinuria.

Because the glomerular mucoid substance noted seemed important in the histologic evaluation of this disease and because histologic methods used in the first experiment did not stain this material, sections fixed in cold acetone were subjected to toluidine blue staining for metachromasia and to Permutt and Johnson's modification[16] of the

Hotchkiss stain. Neither of these methods gave any information about the nature of this pale-staining material which was increased in the glomeruli of animals given nephrotoxic serum.

Experiment 3

Recently it has been noted in this laboratory[17] that older rats on a high fat diet developed marked lipemia and proteinuria 1 week after a single injection of 1 ml. of anti-kidney serum. Fatty lesions of glomeruli and tubules were present at this time, and the glomerular basement membrane was markedly thickened. Smadel[18] and Heymann and Lund[7] have previously noted these fatty lesions and the latter found that the severity of the nephritis was correlated with the amount of fat present in the glomeruli and/or tubules. Furthermore, a review of the literature suggested that the lesions of nephrotoxic "nephritis" are more severe in younger rats and that the *in vivo* manifestations of this disease, such as edema, ascites, and elevated proteinuria, are more marked.

It was reasoned that a high fat diet and large multiple doses of anti-kidney serum in young rats would produce kidney lesions of the most marked degree, which could be studied with relation to the *in vivo* observations and the experimental procedures used.

Plan. Twenty-eight rats weighing 115 to 168 gm. were divided into four groups of 7 rats each. These four groups were placed on a high fat diet composed of the following ingredients per 100 gm.: casein, 18.75 gm.; lard, 30.00 gm.; dextrin, 32.50 gm.; vitamin mixture,* 6.25 gm.; ruffex,† 5.31 gm.; salt mix, 6.25 gm.; choline chloride, 0.625 gm.; cholesterol, 0.31 gm.; oleum percomorphum, 0.5 gtt. Twenty-seven rats weighing 138 to 199 gm. were divided into three groups of 7 rats and one group of 6 rats. They were fed Purina chow *ad libitum*. Two groups of animals on each ration were given seven daily injections of serum and two groups a single injection on the first day. Six rats of each group received anti-kidney serum; the seventh received normal rabbit serum. The dosage schedule varied with the weight of the animal as follows: 100 to 120 gm., 1.1 ml.; 121 to 140 gm., 1.2 ml.; 141 to 170 gm., 1.5 ml.; and 171 to 200 gm., 1.8 ml. Since 9 animals died within 18 hours of the first injection, the dosage was reduced to: 100 to 140 gm., 0.75 ml.; and 141 to 200 gm., 1.0 ml. for the

* Vitamins per 100 gm. of diet furnished by vitamin CHO mix: thiamine, 625 μg.; riboflavin, 1250 μg.; niacin, 1875 μg.; pyridoxine, 625 μg.; calcium pantothenate, 1875 μg.; vitamin K, 1250 μg.

† Fisher Scientific Co.

remainder of the multiple injections. Urine specimens were obtained for protein determinations and microscopic examinations the day before sacrifice except when the schedule was altered by premature death. One experimental animal in each group and 4 control animals were sacrificed at 6 hours. One animal from each group was sacrificed at 1 day, 2 days, 4 days, and 7 days. At 14 days all remaining animals were sacrificed.

Results. It was noted that animals on the high fat diet gained weight at a slower rate than those on chow, but otherwise did well. Control urines were examined from all rats during a 17-day control period during which the above diets were fed. "Normal" proteinuria on these animals ranged from 0.09 mg. per 24 hours per 100 gm. to 2.06 mg. per 24 hours per 100 gm. Rare granular casts were present in the urine of one animal. One animal suddenly stopped breathing and died immediately after injection. It had hydrothorax and pulmonary edema and congestion. One animal died 2 hours after injection, with hydrothorax, pulmonary congestion, ascites, hyperemic liver, and pale kidneys. Six more rats, all in the same group and on Purina chow, died in the next 10 hours with clear or slightly bloody pleural fluid, dark red lungs showing hemorrhage and edema, and kidneys with pale cortex and deep red medulla. Another animal died of overanesthesia at the time of the second injection. It showed 2 ml. of slightly bloody pleural fluid and clear ascites. The kidneys were pale, especially the cortices. One rat given multiple injections died some 6 to 12 hours after the second injection. It had ascites, hydrothorax, dark hemorrhagic lungs, and pale flaccid kidneys. Another multiple injection rat died after the fifth injection, but the tissues were useless because of autolysis. All other animals survived. Many of the animals given multiple injections of anti-kidney serum developed necrosis of their tails, probably resulting from extravasation of small amounts of the anti-kidney serum at the time of injection. Two rats, nos. 66 and 73, showed ascites at the end of the first week. The rat sacrificed then, no. 66, had 20 ml. of clear ascitic fluid. The ascites slowly disappeared in a few days in the other rat and necropsy at 2 weeks showed only 2 ml. of ascitic fluid. Edema was not seen in any of the animals but of the 35 animals sacrificed during this experiment, 6 had hydrothorax and 10 had ascites. Of the 6 animals showing hydrothorax at necropsy, 2 had had a single injection of anti-kidney serum and 4 had had multiple injections. Of the 10 animals showing ascites at necropsy, 3 had had a single injection of anti-kidney serum, and 7 had had multiple injections.

Generally, control rats gained weight and rats with nephritis lost weight, but, as might be expected because of the developing ascites and hydrothorax, there was no correlation between the amount of weight loss and the severity of the nephritis.

Occasional erythrocytes were seen in the urine of rat 47 which died 6 hours after the first injection and showed a significant increase in proteinuria. No other rats showed hematuria. Cylindruria was present in many animals but showed no discernible pattern. The rats on a high fat diet had less proteinuria than those on chow, and those given a single injection of anti-kidney serum tended to reach a peak in urinary protein early in the course of the experiment and then declined appreciably. The proteinuria of the other groups of rats stayed at the high plateau values reached early in the course of the disease (Table III). Multiple injections seemed to enhance the nephrotic picture, but not to a great extent.

Kidney weights, expressed in per cent of the body weight, increased during the experiment in rats receiving anti-kidney serum but not in control rats (Table III). Liver weights expressed in terms of per cent of body weight tended to be greater in rats receiving anti-kidney serum, and splenic weights seemed to vary with the amount of congestion present, but could not be correlated with any other aspect of the "nephritis."

Microscopically, it was not evident why rat 68 died immediately after injection. The only histologic changes noted were mild fatty lesions of the glomeruli and tubules and a mild lipemia. Rat 69, which received a high fat diet and died at 2 hours, had severe pulmonary edema and a marked swelling of the media of small renal arterioles. Rat 62, which was fed chow and died 4 hours after injection, had marked pulmonary hemorrhage and edema and congested glomeruli. The 6 rats (nos. 47, 48, 49, 50, 51, 52) which succumbed 6 to 18 hours after injection were all fed chow and had similar microscopic findings. These consisted of pulmonary edema and hemorrhage, glomerular hemorrhage, and platelet thrombi beginning on the walls of tuft capillaries and gradually filling and distending most of the glomerular capillary loops and even extending into the afferent arterioles in places. With stains using colloidal iron (Hale stain[15]) it was readily appreciated that there was an increase of iron-positive material, presumably mucopolysaccharide, between the thrombosed loops of most glomeruli (Figs. 2 and 3). This was seen also in the animal (no. 68) which died very soon after injection. This material appeared as a pale blue granular mass beginning in the area around the epithelial covering of the

TABLE III

Experiment 3—Urine Protein Values and Kidney Weights

Animal no.*	Anti-kidney serum in ml.			Proteinuria					
				Before injection		After injection			
	Diet	First dose	Later doses†	Urine volume 24 hrs. (ml.)	Protein mg./100 gm./24 hrs.	Day of analysis	Urine volume 24 hrs. (ml.)	Protein mg./100 gm./24 hrs.	Kidney weight as % of body
20	Chow	1.8		10.4	0.52	0§	12.3	34.6	0.85
21	Chow	1.8‡		5.2	0.31	0§	2.4	2.7	0.86
22	Chow	1.8		5.8	1.16	1	6.9	124.0	0.98
23	Chow	1.8		3.6	0.40	4	7.0	186.0	0.97
24	Chow	1.8		1.8	0.42	7	9.0	206.0	1.31
25	Chow	1.8		10.8	0.60	15	12.3	270.0	1.09
26	High fat	1.1		0.0	0.00	0§	1.5	2.0	0.85
27	High fat	1.2‡		0.9	(0.25)¶	0§	3.0	0.4	0.76
28	High fat	1.2		0.7	0.00‖	1	2.4	73.5	0.82
29	High fat	1.2		0.0	0.00‖	2	1.2	54.0	0.88
30	High fat	1.2		3.4	0.21	4	6.0	66.0	0.94
31	High fat	1.5		5.0	0.56	7	3.6	94.0	0.89
32	High fat	1.5		0.0	0.00‖	15	0.9	(20.2)¶	0.92
33	Chow	1.8		2.6	0.29	0§	23.4	120.0	0.75
34	Chow	1.8#		3.2	0.19	1	7.6	4.7	0.81
35	Chow	1.8		9.0	0.56	2	11.3	208.0	0.91
36	Chow	1.8**		5.4	0.30	4	12.8	16.1	0.90
37	Chow	1.8		7.8	0.52	7	7.0	162.5	1.32
38	Chow	1.8		8.2	0.46	14	16.2	226.0	0.97
39	Chow	1.8‡		6.0	0.32	14	8.4	69.0	0.87
40	High fat	1.2		3.0	0.35	0§	3.6	117.0	0.79
41	High fat	1.5		2.0	0.13	1	2.0	48.0	0.72
42	High fat	1.2		1.9	0.50	2	5.5	108.0	0.88
43	High fat	1.5		0.5	0.00‖	4	4.4	73.5	1.09
44	High fat	1.2		8.6	0.62	7	10.0	66.0	1.07
45	High fat	1.5		9.0	0.56	14	15.4	84.5	0.92
46	High fat	1.2‡		3.6	0.48	14	6.2	3.6	0.72
47	Chow	1.8		4.8	0.60	0§	1.8	34.0	
53	Chow	1.8	1.00	2.8	0.33	2	10.0	220.0	0.99
54	Chow	1.8‡	1.00‡	6.6	0.84	15	18.3	8.5	0.90
55	High fat	1.5		1.6	0.09	0§	4.5	54.0	0.85
56	High fat	1.2		1.8	0.13	1	1.9	23.2	0.82
57	High fat	1.2	0.75	1.2	0.14	2	2.7	130.0	1.02
58	High fat	1.5	1.00	3.2	0.85	4	14.0	144.0	1.03
59	High fat	1.2	0.75	1.0	0.13	15	1.5	42.0	
61	High fat	1.5‡	1.00‡	3.2	0.36	15	3.9	1.9	0.70
63	Chow	1.2		5.6	0.40	0§	4.5	163.0	0.79
64	Chow	1.8	1.00	16.0	0.89	1	11.1	233.0	
65	Chow	1.8	1.00	3.6	0.55	2	14.0	300.0	0.92
66	Chow	1.8	1.00	8.8	0.59	4	13.4	224.0	1.11
67	Chow	1.5	1.00	9.0	0.56	15	32.2	300.0	1.23
69	High fat	1.2		11.3	0.52	0	2.4	12.8	
70	High fat	1.2‡	0.75‡	3.5	2.06	0§	8.4	6.4	0.84
71	High fat	1.1	0.75	7.3	0.39	2	6.0	193.0	0.82
72	High fat	1.5	1.00	7.3	0.58	4	17.0	238.0	
73	High fat	1.5	1.00	3.5	2.06	4	0.0	(2.6)¶	0.75
74	High fat	1.2	0.75	2.5	0.22	7	7.6	219.0	1.25

* Animals were numbered consecutively. Omitted numbers indicate premature death without urine samples.

† Rats receiving multiple doses were injected daily if alive, to a maximum of seven injections.

‡ These rats received the indicated amounts of normal rabbit serum rather than anti-kidney serum.

§ These animals had urinalysis 8 hours after initial injection.

‖ No detectable protein with the method used.

¶ Values in parentheses represent determinations when the volume of urine per 24 hours was under 1 ml. It was felt that in these cases the values were not as representative of the true proteinuria as the concentration.

Part of injection escaped subcutaneously.

** Injected intraperitoneally.

tuft and extending up to the capillary basement membrane which was positive to the periodic acid-Schiff stain. Rinehart and Abul-Haj[14] considered this to be acid mucopolysaccharide elaborated by the epithelial cells. Numerous neutrophils were seen in these glomeruli, both intravascularly and extravascularly. The glomeruli were larger than those in the controls. In most animals Hotchkiss positive droplets were seen in the extravascular glomerular space. The tubules showed marked hyaline droplet degeneration and many hyaline casts were evident. Necrosis of tubule cells was not very common.

In general, rats sacrificed at 6 hours had small glomerular capillary lumina. Two animals had evidence of a mild and one a moderate degree of lipemia in the fat stains. Four of the 6 animals had thickening of the part of the glomerular tuft which stained blue with Mallory's azan, red with periodic acid-Schiff's, and buff with the Rinehart-Hale method. We interpreted this area as being endothelial basement membrane as Bell,[19] McManus,[20] and Rinehart, Farquhar, Jung, and Abul-Haj[21] had done (Figs. 4 and 5). Four of these 6 rats also had increased acid mucopolysaccharide. None of the control animals showed these changes. It should be noted that one animal had several small platelet thrombi similar to the massive ones in rats which died early. Acid mucopolysaccharide was increased at this time and the dark blue epithelial basement membrane seemed reduplicated in places. This whole blue area stained faintly with the periodic acid-Schiff stain and was distinct from the dark-staining endothelial basement membrane (Fig. 6). It formed the peripheral part of the area, staining buff with the colloidal iron-cochineal technique (Fig. 5).

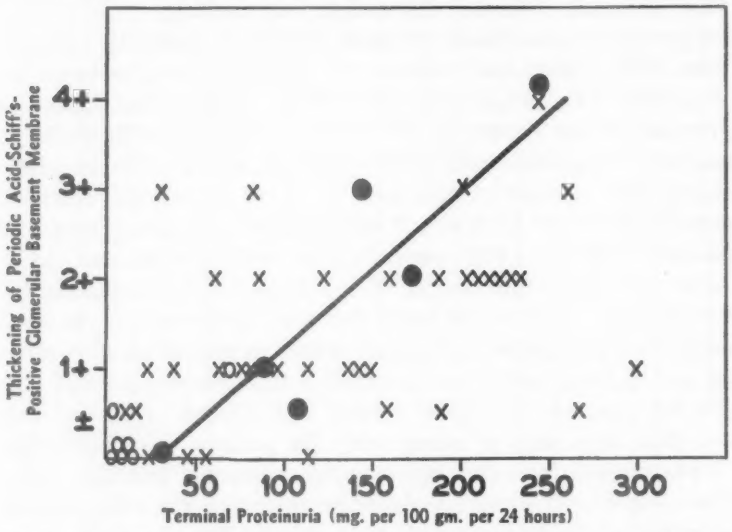
With the passage of time no new types of lesions were demonstrated, but the thin line of acid mucopolysaccharide lining the inside of Bowman's capsule seemed thickened in the same rats which showed an increase in the material between capillary tufts. The lesions increased in severity and while the total number of nuclei present did not appear to increase, relatively more were seen outside the endothelial basement membrane. One could correlate proteinuria equally well with increases in the thickness of the capillary basement membrane which was positive to the periodic acid-Schiff stain and with epithelial acid mucopolysaccaride (Text-figs. 3 and 4). Both changes usually were present to about the same degree in rats which had received nephrotoxic serum.

Although a high fat diet was not necessary to produce fatty glomerular lesions and lipemia, they were found earlier and were more severe in the groups fed this ration (Fig. 7).

Eight of 27 rats fed on chow showed fatty glomerular lesions at sacrifice, while 12 of 28 rats fed a high fat diet showed this change. Fatty lesions in the tubules were thought to be less significant, but they also were more common in the rats receiving the high fat diet.

Discussion

Although the essential nature of the injury to the glomerulus in this disease is still not completely clear, certain points seem evident from

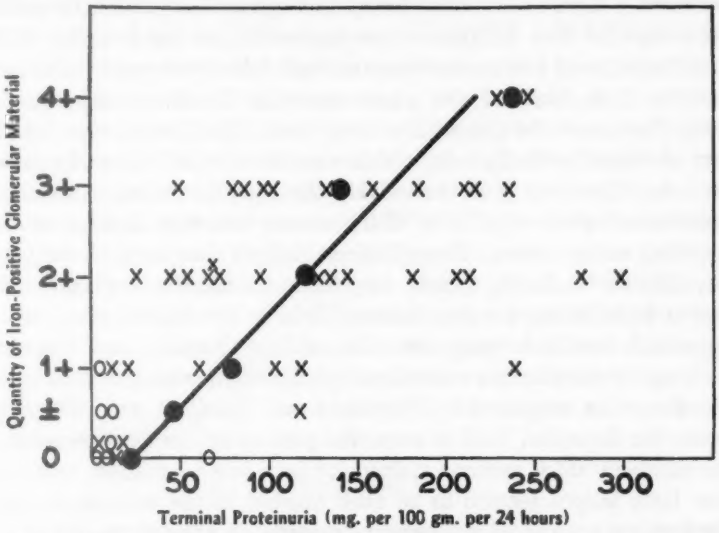


Text-fig. 3. The correlation between the urinary protein excretion at the last determination before death and the average estimated thickening of the periodic acid-Schiff-positive glomerular basement membrane. Experiment 3. Cross indicates anti-kidney serum animals; open circle indicates control animals; solid circle indicates mean points.

this study. Nephrotoxic sera seem to exert an injurious effect on glomerular endothelium as well as epithelium. These changes are particularly well demonstrated in the Hale stains,[15] which apparently have not been applied previously to a study of this experimental disease. With these histologic procedures, endothelial and epithelial elements of the glomerular tufts are easily identified and separated.

Although some observers have described proliferative changes in the glomerular endothelial cells in nephrotoxic nephritis,[3,6,9] these newer staining procedures reveal little evidence of change of this type even after repeated doses of nephrotoxic sera. The essential lesion of the endothelium and the one best correlated with the degree of proteinuria appears to be a swelling of the periodic acid-Schiff-positive basement membrane which goes on to fragmentation and hyaline

droplet degeneration as the process increases in severity. This change is similar to that described by Smadel,[18] Heymann and Lund,[7] Schloss,[5] and others. According to recent studies, anti-kidney serum given intravenously combines with the glomerulus,[22] and the capillary basement membrane is by far the most antigenic part of the glomerulus.[23] Therefore, it would seem probable that anti-kidney serum would combine most readily with this part of the glomerulus and that the changes



Text-fig. 4. The correlation between the urinary protein excretion at the last determination before death and the average estimated increase in iron-positive glomerular epithelial substance as estimated by the modified Hale stain. Experiment 3. Cross indicates anti-kidney serum animals; open circle indicates control animals; solid circle indicates mean points.

which have been observed in the capillary basement membrane may be considered as the primary effect of anti-kidney serum.

The glomerular epithelial changes which occurred quite early after the injection of nephrotoxic serum appeared to be characterized best in the Hale stains.[15] Here the essential change apparently consisted of an increase in iron-positive material which, according to recent interpretation, represents acid mucopolysaccharide.[14] The increase in this material seemed to correlate well with the amount of anti-kidney serum given in those animals receiving multiple doses in the third experiment. In some instances, particularly in the animals sacrificed later in the experiment, it appeared to be accompanied by slight increase in epithelial cell nuclei. But proliferation of epithelial cells was neither constant nor was it ever very severe in these animals. Cres-

cents and synechiae were not seen. That epithelial proliferative changes may become a more important part of the process in very chronic lesions in older animals is suggested by recent observations on 2-year-old rats succumbing 2 to 5 months after receiving two 1 ml. injections of anti-kidney serum intravenously[24] (Fig. 8).

The deposition of fat in the renal glomerulus of rats receiving nephrotoxic serum has been noted by several observers.[7,9,17,18] In experiment 3 these lesions were found to occur earlier in animals receiving a high fat diet. Of the 11 rats succumbing on the first day of the experiment, 4 of 6 rats receiving the high fat diet showed visible glomerular lipid. None of the 5 rats receiving the chow ration showed fatty changes of the glomeruli at that time. This fatty change usually was associated with lipemia, which was observed at this early period after injection only in rats receiving the high fat ration. Later both lipemia and glomerular fatty changes were common findings in rats receiving either ration. These findings suggest that early in the process, lipemia of dietary origin may be more important than the fat depots in initiating the deposition of lipid in the injured glomeruli, a hypothesis not in keeping with that of Farr, Smadel, and Holden.[25] Although these changes seemed to be correlated with the severity of the disease as suggested by Heymann and Lund,[7] it was difficult to locate the deposited lipid in a specific part of the renal glomerulus in the relatively thick sections stained for fat. It is of interest, however, that fatty lesions seemed to be most marked in the animals showing the greatest amount of iron-positive material in their glomeruli.

When large doses of serum were given to young animals, as they were in experiment 3, the rats which did not survive usually died with massive pulmonary edema and hemorrhage, possibly resulting from the combination of anti-kidney serum with the pulmonary vascular structures. Pressman and Eisen[26] have recently demonstrated this type of cross reaction *in vivo* and have suggested that it is the result of common antigens between the vascular elements of kidney and lung. In addition to the pulmonary changes, these rats had glomerular thrombosis and hemorrhage. One may assume that if the animals had survived this severe damage for several days, fibrosis of thrombosed loops, which has been described,[9] would occur. None of the animals in these experiments showed glomerular fibrosis.

In these experiments ascites was never seen in rats weighing 200 gm. or more and was rare in the younger and smaller rats used in the third experiment. Edema was never seen. This agrees with the observations by Lippman, Marti, and Campbell[8] who used rats weighing about 150 gm. However, investigators using rats weighing 50 to 80

gm.[4,6,7] have reported seeing ascites and/or edema frequently. From these observations it is evident that the appearance of ascites and/or edema is at least partly dependent on the age and weight of the rats used. This may be related to the observation that glomerular lesions in younger rats are mainly degenerative rather than proliferative and hence a nephrotic picture is more likely to be produced. It suggests that the capacity for glomerular epithelial proliferation is poor in the young rat and increases only slightly with age.

The observations in this study are compatible with the view expressed by other workers[7,9] that the early lesions and the clinical state produced by the injection of nephrotoxic serum into the rat and particularly into the young rat are most similar to those seen in human lipid nephrosis. This is in contrast to the lesions produced in the rabbit by anti-kidney serum, which are more likely to result in marked proliferative changes and azotemia.[9,27-29] Both are to be contrasted with the experimental nephritis resulting when rabbits are injected with large doses of a foreign serum or gamma globulin.[9,30-33] Quantitative studies of these other experimental processes with varying dosage and time between injection and sacrifice are indicated. It seems likely that if they were studied using the newer staining procedures which are now available, one might be better able to relate experimental nephritis to human renal disease.

Summary

Descriptions of the clinical and pathologic picture resulting from the injection of nephrotoxic serum into rats have varied. Resemblance to human glomerulonephritis has impressed some observers; others have compared the disease with lipid nephrosis.

Three short-term experiments were performed which permitted study of nephrotoxic nephritis, and of the influence on the disease of diet, weight of animals used, dosage of nephrotoxic serum injected, and duration of the induced state.

When an excessive dose of nephrotoxic serum was given to a rat, it usually manifested hematuria and elevated proteinuria, and died acutely with pulmonary edema and hemorrhage. Glomerular hemorrhage and thrombosis, accompanied by a neutrophilic infiltrate, were seen, as well as an increase in glomerular acid mucopolysaccharide. Many hyaline casts were present, but necrosis of tubule cells was not common.

When doses of nephrotoxic serum which were compatible with life were injected, marked proteinuria and rare hematuria developed. Proteinuria increased with the quantity of serum injected within certain

limits. Both periodic acid-Schiff's-positive endothelial basement membrane and acid mucopolysaccharide associated with glomerular epithelium were increased. The thickening of the former was accompanied by fragmentation and in the more severe instances by separation of the material into droplets or clumps. Proliferation was not seen. The intensity of these changes could be correlated well with the degree of proteinuria at the time of sacrifice.

The rats which showed early increases in urinary protein and which declined to within the limits of control values at the time of sacrifice had normal appearing glomeruli and tubules. Thus, the renal lesions appeared to be reversible.

The rats receiving high fat diets which were injected with anti-kidney serum showed lipemia and fatty lesions of glomeruli and tubules 1 day after injection. Rats receiving Purina chow diets and similarly injected did not show the above changes for several days. Lipemia seemed to favor the uptake of fat by injured cells. The rats receiving the high fat diet had less proteinuria than those on chow. Multiple injections of anti-kidney serum into rats produced a peak in urinary protein levels early in the experiment. These levels remained high. The proteinuria in rats receiving a single injection declined appreciably with the passage of time.

A review of the literature indicates that when rats weighing less than 100 gm. were injected with anti-kidney serum in large doses, they commonly developed ascites and marked edema. In the present study large injections of anti-kidney serum were given both younger and older rats. Results indicate that thickening of glomerular endothelial basement membrane and increase in glomerular acid mucopolysaccharide were more severe in younger animals. Both age and weight, at least in part, seemed to determine the nature of the disease produced.

From the clinical and pathologic observations it is concluded that the acute disease produced in rats can resemble either nephrotic nephritis or "pure" lipid nephrosis depending on the severity of the process. Glomerular changes can be relatively slight at this stage and yet proteinuria may be present. The chronic disease induced by the same procedure resembles chronic glomerulonephritis with nephrotic clinical manifestations.

We wish to acknowledge the assistance of Dr. Tom Johnson, Dr. Sylvan Rapheal, and Dr. Harry Williams in preparing the anti-kidney serum used in these experiments.

The photomicrographs were made by Jean M. Crunelle of the Photography Department of the University of Chicago Clinics.

REFERENCES

1. Lindemann, W. Sur le mode d'action de certains poisons rénaux. *Ann. Inst. Pasteur*, 1900, 14, 49–59.
2. Woltmann, H. A study of the changes in the blood and blood-forming organs produced by cytotoxic sera, with special reference to haemolymphotoxin. *J. Exper. Med.*, 1905, 7, 119–162.
3. Masugi, M. Über das Wesen der spezifischen Veränderungen der Niere und der Leber durch das Nephrotoxin bzw. das Hepatotoxin. Zugleich ein Beitrag zur Pathogenese der Glomerulonephritis und der eklamptischen Lebererkrankung. *Beitr. z. path. Anat. u. z. allg. Path.*, 1933, 91, 82–112.
4. Smadel, J. E. Experimental nephritis in rats induced by injection of anti-kidney serum. I. Preparation and immunological studies of nephrotoxin. *J. Exper. Med.*, 1936, 64, 921–942.
5. Schloss, G. Der Regulationsapparat am Gefässpol der Nierenkörperchens bei der Masugi-Nephritis der Ratte. *Helvet. med. acta*, 1945, 12, 777–800.
6. Solomon, D. H., Gardella, J. W., Fanger, H., Dethier, F. M., and Ferrebee, J. W. Nephrotoxic nephritis in rats. Evidence for the glomerular origin of the kidney antigen. *J. Exper. Med.*, 1949, 90, 267–272.
7. Heymann, W., and Lund, H. Z. Nephrotic syndrome in rats. *Pediatrics*, 1951, 7, 691–706.
8. Lippman, R. W., Marti, H. U., and Campbell, D. H. Nephrotoxic globulin nephritis. I. Course after a single intravenous injection. *A. M. A. Arch. Path.*, 1952, 53, 1–14.
9. Ehrich, W. E., Forman, C. W., and Seifer, J. Diffuse glomerular nephritis and lipid nephrosis: correlation of clinical, morphological, and experimental observations. *A. M. A. Arch. Path.*, 1952, 54, 463–503.
10. Hektoen, L., and Welker, W. H. Precipitin production in rabbits following intramuscular injection of antigen absorbed by aluminum hydroxide. *J. Infect. Dis.*, 1933, 53, 309–311.
11. Kolmer, J. A., and Boerner, F. Approved Laboratory Technic. D. Appleton-Century Co., New York, 1941, ed. 3, pp. 137–139.
12. Bensley, R. R., and Bensley, S. H. Handbook of Histological and Cytological Technique. University of Chicago Press, Chicago, 1938, p. 75.
13. Hotchkiss, R. D. A microchemical reaction resulting in staining of polysaccharide structures in fixed tissue preparations. *Arch. Biochem.*, 1948, 16, 131–141.
14. Rinehart, J. F., and Abul-Haj, S. K. An improved method for histologic demonstration of acid mucopolysaccharides in tissues. *A. M. A. Arch. Path.*, 1951, 52, 189–194.
15. Hale, C. W. Histochemical demonstration of acid polysaccharides in animal tissues. *Nature, London*, 1946, 157, 802.
16. Permutt, S., and Johnson, F. B. Histochemical studies on the lens following radiation injury. *A. M. A. Arch. Path.*, 1953, 55, 20–30.
17. Wissler, R. W., Collins, J. L., Schroeder, M. A., and Soules, K. Unpublished observations.
18. Smadel, J. E. Experimental nephritis in rats induced by injection of anti-kidney serum. III. Pathological studies of the acute and chronic disease. *J. Exper. Med.*, 1937, 65, 541–555.
19. Bell, E. T. The pathology and pathogenesis of clinical acute nephritis. *Am. J. Path.*, 1937, 13, 497–552.
20. McManus, J. F. A. Medical Diseases of the Kidney. Lea & Febiger, Philadelphia, 1950, 176 pp.

21. Rinehart, J. F., Farquhar, M. G., Jung, H. C., and Abul-Haj, S. K. The normal glomerulus and its basic reactions in disease. *Am. J. Path.*, 1953, **29**, 21–31.
22. Pressman, D., Eisen, H. N., Siegel, M., Fitzgerald, P. J., Sherman, B., and Silverstein, A. The zone of localization of antibodies. X. The use of radioactive sulfur 35 as a label for anti-kidney serum. *J. Immunol.* 1950, **65**, 559–569.
23. Krakower, C. A., and Greenspon, S. A. Localization of the nephrotoxic antigen within the isolated renal glomerulus. *A. M. A. Arch. Path.*, 1951, **51**, 629–639.
24. Wissler, R. W., Eilert, M. L., Schroeder, M. A., and Cohen, L. Unpublished observations.
25. Farr, L. E., Smadel, J. E., and Holden, R. F., Jr. Observations on occurrence of lipemia in rats with nephrotoxic nephritis. *Proc. Soc. Exper. Biol. & Med.*, 1942, **51**, 178–179.
26. Pressman, D., and Eisen, H. N. Specific localization of anti-rat lung-serum in the lung. *Proc. Soc. Exper. Biol. & Med.*, 1950, **73**, 143–146.
27. Masugi, M. Über die experimentelle Glomerulonephritis durch das spezifische Antinierenserum. Ein Beitrag zur Pathogenese der diffusen Glomerulonephritis. *Beitr. z. path. Anat. u. z. allg. Path.*, 1933–34, **92**, 429–466.
28. Arnott, W. M., Kellar, R. J., and Matthew, G. D. Experimental glomerulonephritis produced by the use of specific sera. *Edinburgh M. J.*, 1936, **43**, 233–242.
29. Kay, C. F. The mechanism of a form of glomerulonephritis. Nephrotoxic nephritis in rabbits. *Am. J. M. Sc.*, 1942, **204**, 483–490.
30. Rich, A. R., and Gregory, J. E. The experimental demonstration that periarteritis nodosa is a manifestation of hypersensitivity. *Bull. Johns Hopkins Hosp.*, 1943, **72**, 65–88.
31. Hawn, C. V., and Janeway, C. A. Histological and serological sequences in experimental hypersensitivity. *J. Exper. Med.*, 1947, **85**, 571–590.
32. Wissler, R. W., Smull, K., and Lesh, J. B. The effects of various horse serum fractions in producing cardiovascular and renal lesions in rabbits. *J. Exper. Med.*, 1949, **90**, 577–594.
33. More, R. H., and Waugh, D. Diffuse glomerulonephritis produced in rabbits by massive injections of bovine serum gamma globulin. *J. Exper. Med.*, 1949, **89**, 541–554.

LEGENDS FOR FIGURES

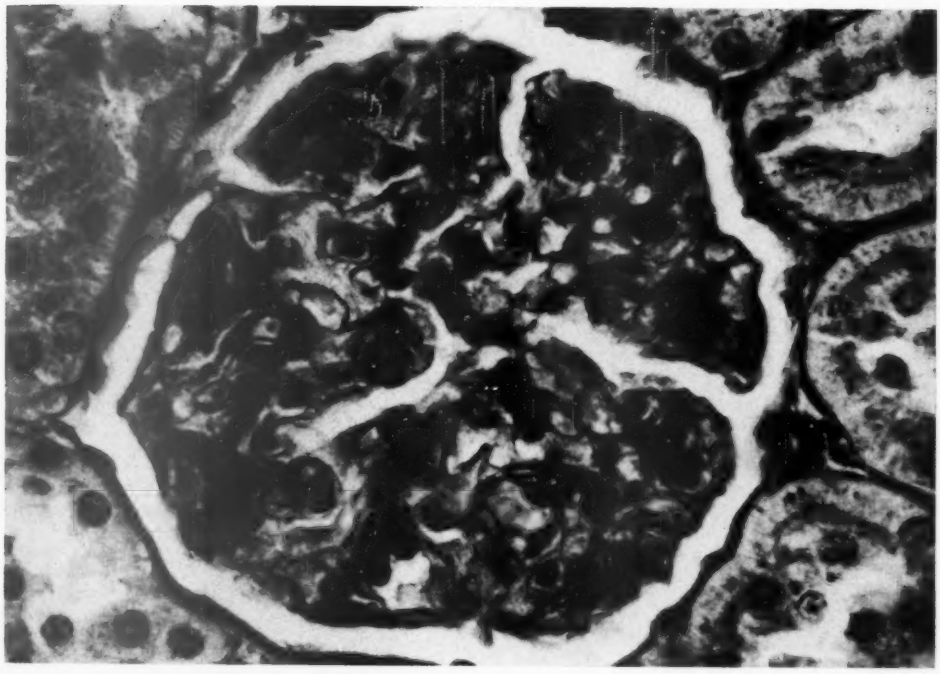
FIG. 1. Glomerulus of animal 17A which excreted urinary protein at the rate of 43.5 mg. per 24 hours per 100 gm., 14 days after injection with 1.5 ml. of anti-kidney serum. It was sacrificed the next day. The periodic acid-Schiff-positive capillary basement membrane is focally thickened and fragmented. Capillary loops are separated by mucopolysaccharide that does not stain by this method. Periodic acid-Schiff's stain. × 845.

FIG. 2. Glomerulus of control animal 21 which excreted urinary protein at the rate of 2.7 mg. per 24 hours per 100 gm., 6 hours after injection with 1.8 ml. of normal rabbit serum. It was sacrificed at that time. The endothelial basement membrane is thin. Capillary loops are almost contiguous and have little acid mucopolysaccharide between them. Colloidal iron combined with periodic acid-Schiff's stain. × 845.

1

2

1



2

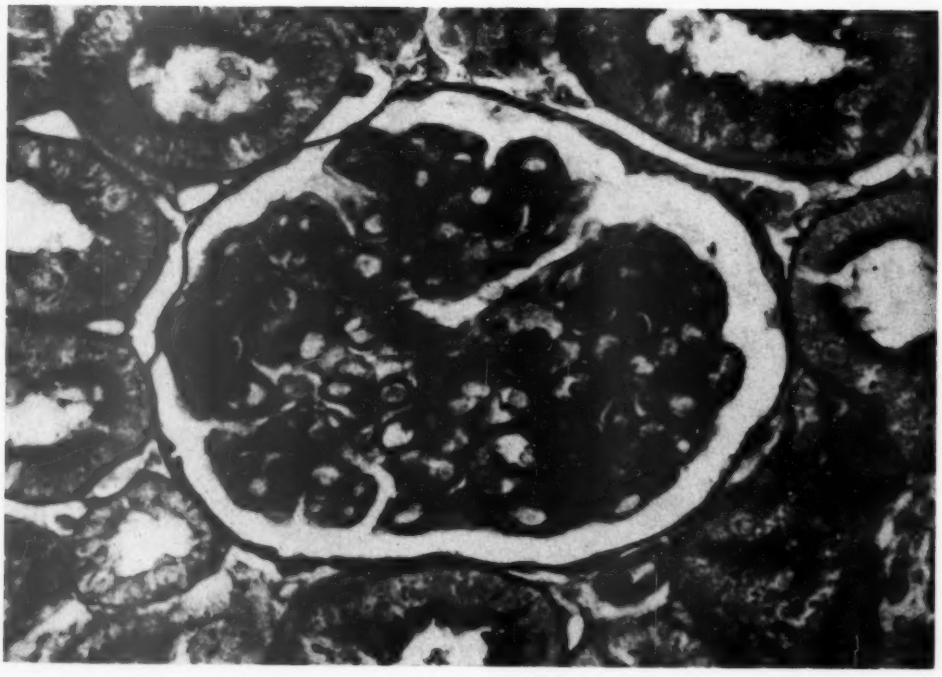
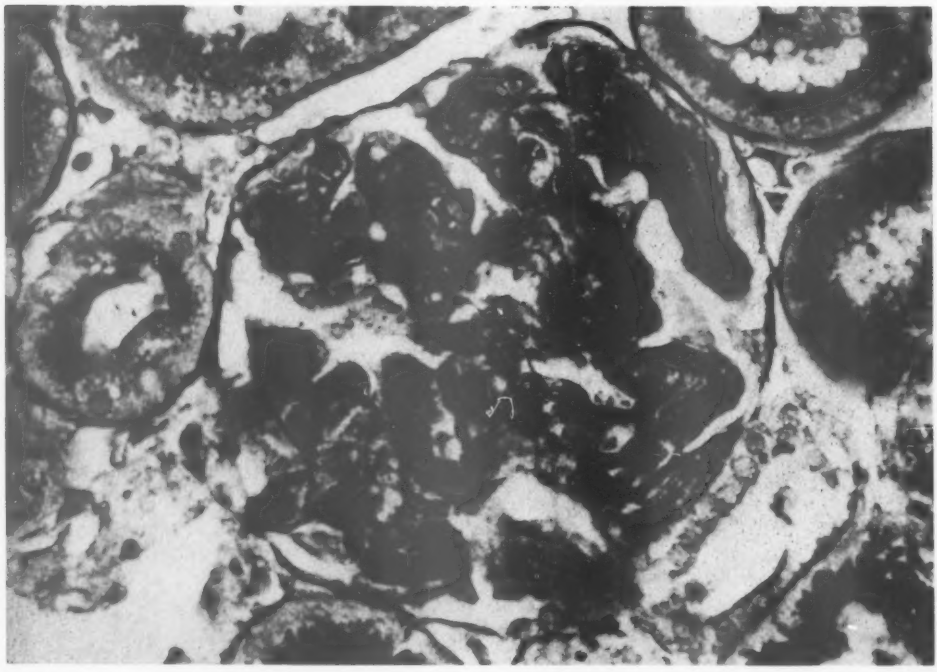
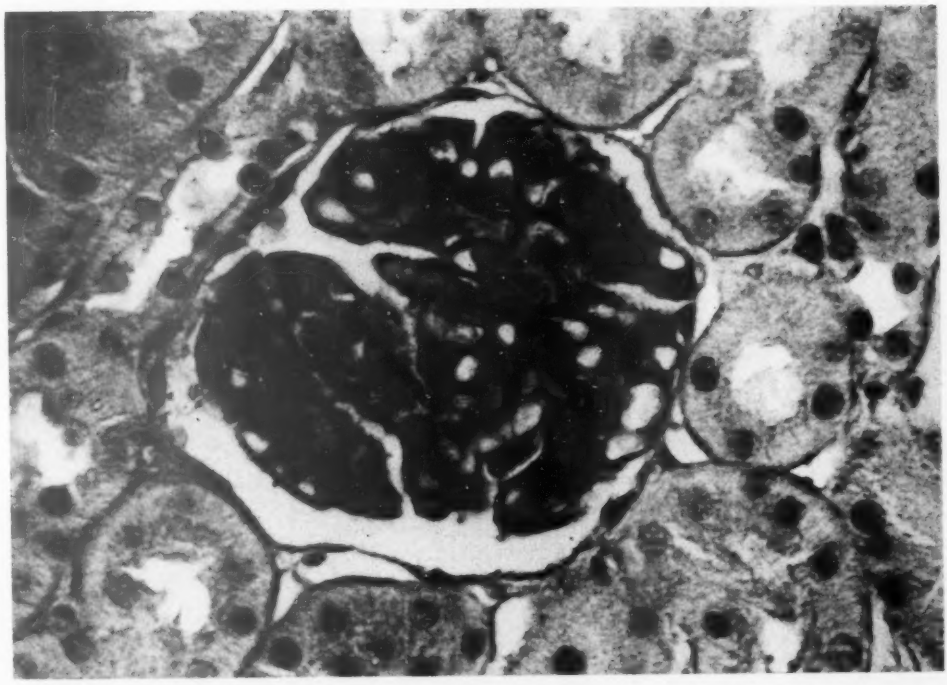


FIG. 3. Glomerulus of animal 47 which excreted urinary protein at the rate of 34.0 mg. per 24 hours per 100 gm., 6 hours after injection with 1.8 ml. of anti-kidney serum. It died 6 hours after injection of massive pulmonary edema and hemorrhage. Glomerular hemorrhage was present. Hyaline droplet degeneration of glomerulus and adjacent tubule is evident. Platelet thrombi focally obstructing the capillary tuft lumina can be seen. Colloidal iron combined with periodic acid-Schiff's stain. × 845.

FIG. 4. Glomerulus of control animal 27 which excreted urinary protein at the rate of 0.43 mg. per 24 hours per 100 gm. upon sacrifice 6 hours after injection with 1.2 ml. of normal rabbit serum. Capillary loops are well filled with erythrocytes and appear contiguous, with little acid mucopolysaccharide between. Colloidal iron combined with cochineal stain. × 845.

FIG. 5. Glomerulus of animal 24 which excreted urinary protein at the rate of 206 mg. per 24 hours per 100 gm. upon sacrifice 7 days after injection with 1.8 ml. of anti-kidney serum. Capillary lumina are difficult to see because of decreased diameter. Material seen between adjacent lumina consists of buff-staining endothelial basement membrane and blue-staining acid mucopolysaccharide associated with the thickened epithelial basement membrane. Colloidal iron combined with cochineal stain. × 845.





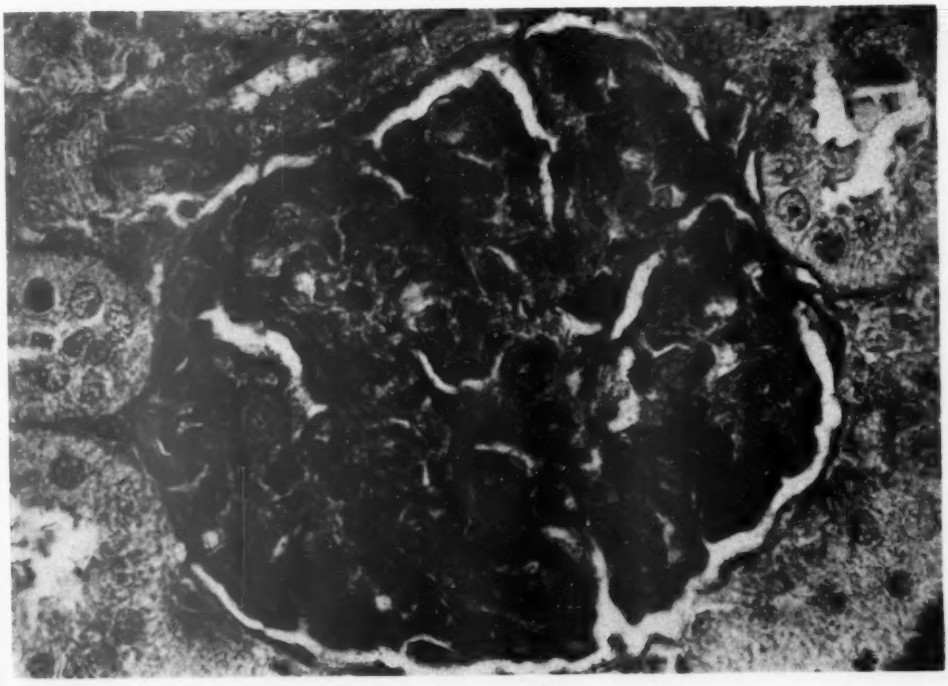
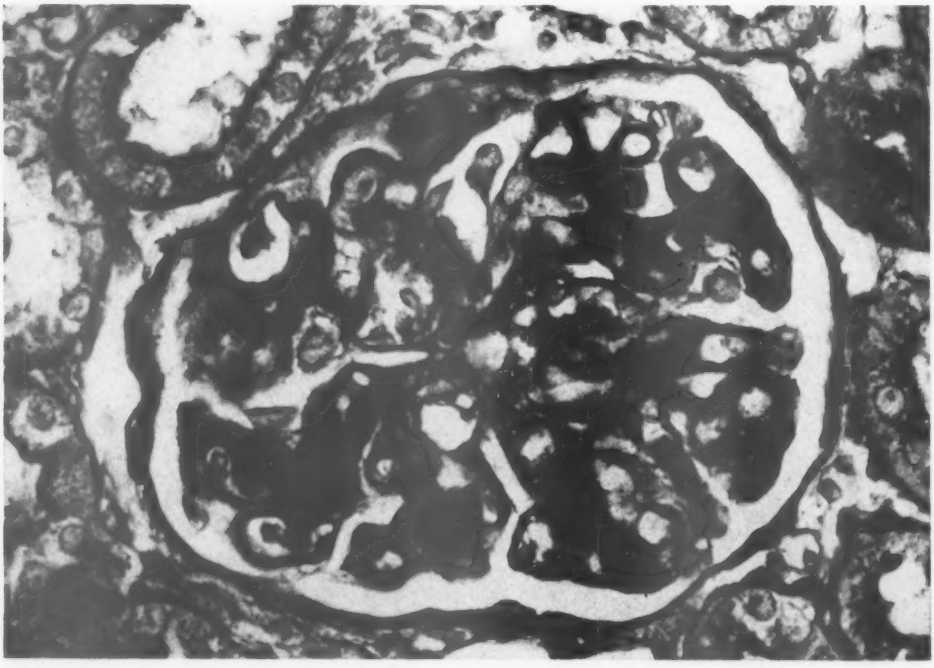
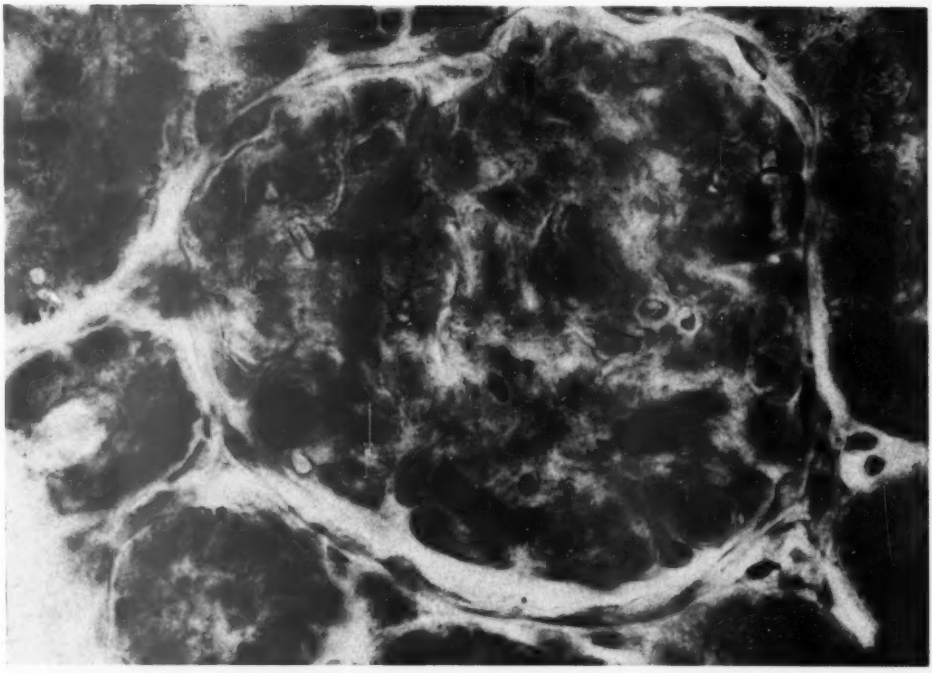


FIG. 6. Glomerulus of animal 67 which excreted urinary protein at the rate of 300 mg. per 24 hours per 100 gm. upon sacrifice 15 days after anti-kidney serum injections were begun. It received 1.5 ml. on the first day and 1.0 ml. daily for 6 days. The periodic acid-Schiff-positive endothelial basement membrane is thickened and has undergone some fragmentation. Pale-staining acid mucopolysaccharide is increased and can be seen between most of the capillary loops. Colloidal iron combined with periodic acid-Schiff's stain. × 845.

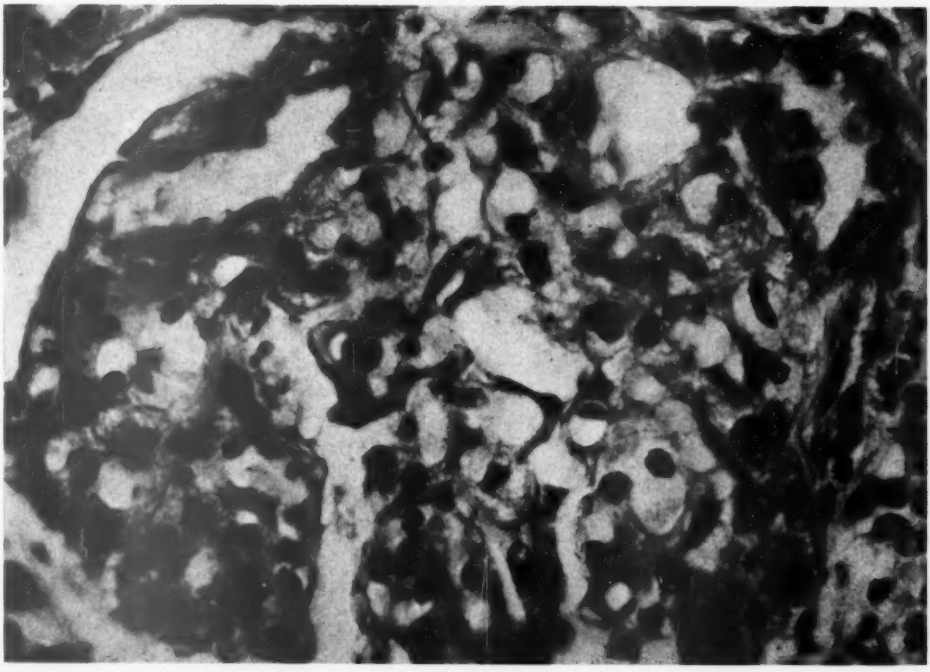
FIG. 7. Glomerulus of animal 58 which was on a high fat ration and excreted urinary protein at the rate of 144 mg. per 24 hours per 100 gm. upon sacrifice 4 days after anti-kidney serum injections were begun. It received 1.5 ml. on the first day and 1.0 ml. on the next 2 days. Both glomerular and tubular cells contain much fat. No such lesions were seen this early in kidneys of rats on Purina chow. Frozen section, Sudan IV stain. × 845.

FIG. 8. Glomerulus of animal 232. It was given two intravenous anti-kidney serum injections of 1 ml. each, and fed on a high fat, high choline diet. It succumbed 23 weeks and 5 days later. The glomerulus is greatly enlarged and distorted. Synechiae are present. The endothelial basement membrane is thickened and increased numbers of nuclei are present. Hematoxylin and eosin stain. × 845.





7



8

LEUKEMIA IN GUINEA-PIGS *

CHARLES C. CONGDON, M.D., and EGON LORENZ, PH.D.

(From the National Cancer Institute, National Institutes of Health, U.S. Public Health Service, Bethesda, Md., and the Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Ill.)

During the past 10 years, hematologic and survival studies[1,2] of irradiated and non-irradiated guinea-pigs have revealed at the National Cancer Institute 10 cases of primary lymphatic leukemia. The present paper deals chiefly with the incidence, the findings in the peripheral blood, and the morphologic lesions found at necropsy of these primary cases. The general characteristics of transplantable leukemia in the guinea-pig are also described.

HISTORICAL ACCOUNT

Nearly 40 years ago, Dickson[3] found a large, freely movable tumor mass on the ventral surface of the neck of a male guinea-pig. Around the main tumor mass and in the right axilla he observed some smaller tumor nodules. Microscopically both the main tumor and the associated nodules were composed of cells resembling lymphocytes. Miguez[4] discovered a large subcutaneous tumor in the region of the thyroid gland in a female guinea-pig. The tumor cells resembling lymphocytes had infiltrated the liver and kidneys. Miguez did not describe the bone marrow, lymph nodes, or peripheral blood. Transplantation of the tumor to the rat, mouse, and rabbit failed, but in guinea-pigs it was successful through its fourth transplant generation. Fischer and Kantor[5] continued the transplantation of Miguez' tumor through 50 passages, and considered it to be a lymphosarcoma. In 1925 Bender[6] described a lymphosarcoma involving the left ventricle of the heart in a male guinea-pig. Snijders[7,8] found generalized lymphadenopathy, splenomegaly, and hepatomegaly at necropsy of a guinea-pig in Sumatra. The thymus and the lymphatic tissue of the intestine also were enlarged. The blood showed a marked increase in leukocytes. Microscopically the involved tissues were extensively invaded by "primitive blood or lymph" cells. A transplant of the tumor grew in one guinea-pig which died of leukemia within 90 days, with a leukocyte count of 67,600 cells.[7-9] In Sumatra, Snijders' tumor was carried to the 62nd passage with about 80 per cent takes. At the 54th passage of this

* Presented, in part, at the Fiftieth Annual Meeting of the American Association of Pathologists and Bacteriologists, St. Louis, April 4, 1953.

Received for publication, July 20, 1953.

tumor a second transplant series was started in Holland, and ran through 19 passages with about 35 per cent takes. The latter series was eventually lost.[10] Four distinct morphologic variations in the tumor were observed during transplantation. These were: giant cell lymphoblastic leukemia, aleukemic lymphadenosis, Sternberg's leukosarcomatosis, and a lymphosarcoma resembling that of man. Snijders believed, however, that these variations were secondary features of the major neoplastic process. In the leukemic state the white blood cell count sometimes reached several hundred thousand cells per cubic millimeter. Throughout the course of transplantation of Snijders' tumor, the cell type remained that of a primitive lymphocyte. Transplantation was possible by transfer of leukemic blood, emulsions of lymph nodes, spleen, thymus, bone marrow, or of pleural or peritoneal fluid that contained leukemic cells. Six of Snijders' guinea-pigs bearing the leukemia were pregnant. At necropsy, the fetuses failed to show evidence of leukemia. Gerlach[11] found that the slower the course of leukemia in guinea-pigs, the more advanced the pathologic changes became, and that these changes may be absent in cases of short duration. Ball and Pagnon[12] observed a round cell sarcoma of the kidney in a guinea-pig. The tumor weighed 140 gm., and completely destroyed the right kidney. It is difficult to determine whether this case should be classified with the tumors of hematopoietic origin. Lorenz *et al.*[1] mentioned 3 cases of leukemia in a series of irradiated and 2 cases in non-irradiated guinea-pigs. Rogers[13] reported a lymphoblastoma in a senile animal of a tumor-susceptible family of guinea-pigs. Heston and Deringer[14] found 2 cases of leukemia in 51 guinea-pigs of strain 2 that had been treated with intravenous injections of 20-methylcholanthrene or 1,2,5,6-dibenzanthracene. They described one case of leukemia and one tumor of blood-forming tissues (probably a reticulum-cell sarcoma) in male animals of 60 guinea-pigs of strain 13 examined. Their tumors did not occur at "sufficiently high frequency to be considered as induced," although none of the controls had leukemia.

Description of Ten Cases of Leukemia

Incidence of Leukemia in Control and Irradiated Guinea-Pigs

Ten cases of leukemia occurred in a series of 303 guinea-pigs 12 months of age or older. Of these, 112 were of strain 2, 30 of strain 13, and 161 were heterogeneous hybrids. Approximately one half were male. Table I shows the distribution and incidence of leukemia in the control and irradiated groups. The sex and strain of the leukemic guinea-pigs are given in Table II.

TABLE I

Incidence of Lymphatic Leukemia in Control and Irradiated Guinea-Pigs

Group	Number of animals 12 months or older dying in each 6 month period*												Totals	Leukemia	Remarks
	12–18	18–24	24–30	30–36	36–42	42–48	48–54	54–60	60–66	66–72	72–78	78–84		%	
Control	0/6	0/5	0/14	0/2	1/9	1/10	0/9	0/14	1/6	1/2	0/1	0/2	4/80	5.0	Includes: 55 non-irradiated animals; 12 that received one acute x-irradiation of either 15 or 55 r.†; 17 receiving chronic total body gamma irradiation of 0.11 r., daily (8 hrs.) during entire life span†
X-irradiation	0/12	0/12	1/9	1/9	0/5	0/1							2/48	4.2	220 r. to 420 r. acute total body irradiation at 2 to 3 months of age
Chronic gamma irradiation	0/24	0/16	0/21	1/16	1/11	0/11	0/6	0/4	0/2	0/1	0/1		2/113	1.8	Received 1.1, 2.2, 4.4, or 8.8 r. daily (8 hrs.) during entire life span
Chronic gamma irradiation (limited exposure)	1/10	0/5	0/6	1/6	0/13	0/3	0/7	0/2					2/52	3.8	Received limited exposures of either 4.4 r. (for 12 to 18½ months) or 8.8 r. (for 3 to 8 months)

* Expressed as: $\frac{\text{animals necropsied with leukemia}}{\text{animals necropsied without leukemia}}$

† These animals are included in the control group since it is believed that the radiation exposures were relatively insignificant.

Microscopic examination of the tissues of all 303 guinea-pigs was made to determine the presence or absence of leukemia. Since an additional large number of irradiated and control guinea-pigs are still living, the results obtained at this date may require modification after the death of these animals.

Peripheral Blood Studies in Primary Leukemia

Blood cell counts were taken periodically on 9 of the 10 leukemic guinea-pigs from the time they were first received in the laboratory

TABLE II

Sex and Strain of Ten Leukemic Guinea-Pigs

	Strain 2	Strain 13	Heterogeneous hybrids
Male	1 (Control)	1 (Irradiated)	1 (Irradiated)
Female	3 (1 Control) (2 Irradiated)	1 (Irradiated)	3 (2 Control) (1 Irradiated)

at 2 to 3 months of age until death. The frequency of the counts varied from once a month for control animals, once every 2 weeks for irradiated animals, to three times a week for critically ill animals. The counts routinely included white blood cells, differential leukocytes, red blood cells, and platelets. Hemoglobin values also were determined. The 9 cases of leukemia occurred in different experimental groups of animals (5 to 11 in a group), and peripheral blood data are available for comparison with each leukemic guinea-pig. Table III shows the chief hematologic findings near the time of death for each leukemic animal. Leukemic cells in the peripheral blood were not separated from lymphocytes in making the differential count. The number of Kurloff cells was the same in the leukemic and comparable control animals. The structure of the leukemia cells in Wright's preparations of peripheral blood was remarkably similar in all 9 cases of leukemia. They were large round cells with round and sometimes indented nuclei (Fig. 1). The cells varied in size, and in one case (no. 2) the diameter of the leukemic cells in a blood smear ranged from 9.4 to 13 μ with an average diameter of 11.4 μ. The nuclear chromatin was less dense than that of the normal small lymphocytes. The arrangement of the chromatin was loose and lace-like with one or two condensed areas. Nucleoli were not revealed by Wright's stain. The cytoplasm of the leukemia cell was small in amount and colored blue. Occasional small

TABLE III

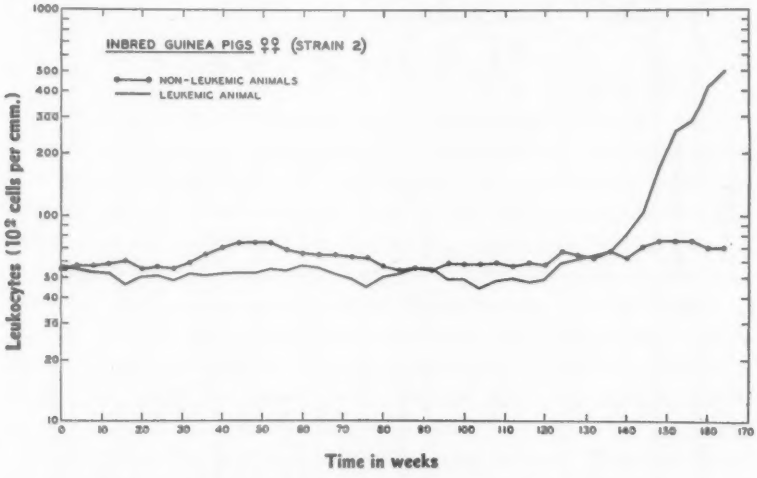
Peripheral Blood Cell Counts Near Time of Death and Irradiation Dosage in Guinea-Pigs with Lymphatic Leukemia

Animal case number	Sex and strain	Age at death	Total body irradiation	White blood cell count	Polymorphonuclear leukocytes	Leukemic cells and lymphocytes	Red blood cell count	Platelets	Approximate duration of leukemic blood picture
		mos.			%	%	*millions*		
1	Male (2)	47	None	104,800	8	90	4.98	128,700	6½ mos.
2	Female (not inbred)	70½	None	53,600	7	92	3.87	85,800	4½ mos.(?)
3	Female (2)	40½	None	55,000	39	58	2.98	265,000	1½ mos.
4	Female (2)	27	400 r. acute x-irradiation at 2 to 3 mos. of age	*	*	*	*	*	*
5	Male (not inbred)	35	310 r. acute x-irradiation at 2 to 3 mos. of age	54,600	26	74	1.34	60,500	1 mo.
6	Female (not inbred)	63	0.11 r. gamma irradiation daily (8 hrs.) for 58½ mos., total dose 200 r.	16,850	63	28	4.66	169,400	Few days
7	Female (not inbred)	37¼	2.2 r. gamma irradiation daily (8 hrs.) for 31¼ mos. plus 2 acute x-irradiations of 55 r. each; total dose, 2100 r.	238,700	2	98	2.31	73,700	3½ mos.
8	Female (2)	30¾	2.2 r. gamma irradiation daily (8 hrs.) for 27½ mos.; total dose, 1850 r.	61,300	16	81	3.19	20,900	Few days
9	Female (13)	31½	4.4 r. gamma irradiation daily (8 hrs.) for 18½ mos.; dose limited to 2600 r.; no irradiation last 10 mos. of life	121,600	2	97	4.72	269,500	Few days to 4 weeks
10	Male (13)	13¼	8.8 r. gamma irradiation daily (8 hrs.) for 8 mos.; dose limited to 2450 r.; no irradiation last 3 mos. of life; received bone marrow on cessation of irradiation	25,350		100	2.20	33,000	10 days

* No blood counts during life; leukemic blood at necropsy.

vacuoles were present. The leukemia cell closely resembled the normal lymphoblast.

Charts of the blood cell counts were prepared for each of the 9 leukemic guinea-pigs. In these, running averages of the total leukocyte and lymphocyte counts of each leukemic animal were compared with similar running averages for the other non-leukemic animals of the same group as the particular leukemic guinea-pigs. Such charts gave a view of these hematologic findings over nearly the entire life span of the animal. At the same time, the counts of a group of animals that did not develop leukemia were available for comparison. Analysis of these charts showed that the peripheral blood of 6 of the leukemic guinea-pigs was similar to that of the non-leukemic animals until leukemia developed. Text-figure 1 shows this in the case of animal 1. In



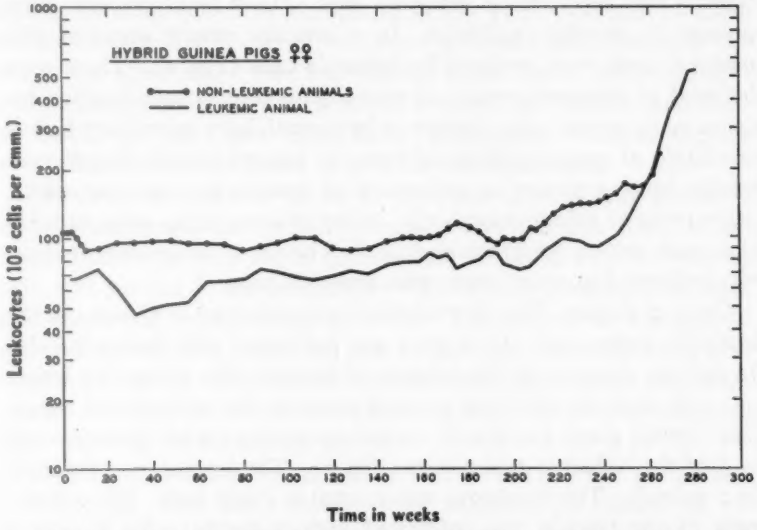
Text-fig. 1. Running averages of the total leukocyte count of a guinea-pig from 2 to 3 months of age until leukemia developed after about 135 weeks. This is compared with the combined running averages of leukocyte counts of a small series of guinea-pigs, observed during the same period, that did not develop leukemia.

one leukemic guinea-pig (no. 5), the blood data on the non-leukemic group were inadequate for this type of analysis. In 2 of the leukemic guinea-pigs (nos. 2 and 7), the analysis showed over a long span of time significant differences between the guinea-pigs that subsequently developed leukemia and those that did not.

In leukemic guinea-pig 2, the total leukocyte and the lymphocyte counts were consistently depressed throughout most of its life span. Text-figure 2 shows this feature for the total leukocyte count. The

terminal rise in the curve for the 11 non-leukemic animals was probably the result of infection in the 2 animals of this group that survived longest.

The blood chart for leukemic animal 7 showed a significantly elevated total leukocyte and lymphocyte count for 85 weeks prior to the detection of leukemic blood. These counts averaged about 3,000 cells per cmm. higher than the average counts of 8 non-leukemic guinea-pigs similarly treated.



Text-fig. 2. Running averages of the total leukocyte count of a guinea-pig from 2 to 3 months of age until the appearance of leukemia. The depression of the leukocyte count throughout the life span of this animal in comparison with the non-leukemic group was unique.

Morphologic Findings in the Leukemic Guinea-Pigs

The general features of a serious illness with ruffling of the hair, pallor of mucous membranes, sluggishness of response, and loss of luster of the eye were noted in some of the leukemic animals. Greatly enlarged peripheral lymph nodes were noted in a few before death. Weight charts of the entire group of 10 leukemic guinea-pigs throughout their life span showed no particular characteristics that differed from those of the non-leukemic animals. At necropsy, the 10 animals were remarkably similar.

Brain and Spinal Cord. The brain and cord were examined in only 2 of the leukemic guinea-pigs, and they appeared to be normal on gross examination. However, microscopically it was found that the lepto-

meninges of both the brain and the lumbar region of the spinal cord were slightly infiltrated with leukemia cells.

Thorax. The heart appeared to be normal upon gross observation. The pericardial adipose tissue at the base of the heart of all 10 leukemic animals was moderately infiltrated with leukemia cells. The mediastinal lymph nodes were in every case enlarged and replaced by leukemia cells. Gross study revealed no significant alteration of the lungs. Microscopically the lymphatic tissue around the blood vessels and bronchi was replaced by leukemia cells. Many leukemia cells were present in alveolar capillaries. In 3 animals, patchy areas of pulmonary tissue were replaced by leukemia cells (Fig. 2). These were believed to represent regions of pre-existing chronic inflammation because many guinea-pigs, thought to be normal, have pulmonary lesions consisting of patchy subpleural areas of atelectasis and fibrosis containing large perivascular collections of lymphocytes and sometimes other types of inflammatory cells. Many of these areas have, in addition, rudimentary lymphatic nodules.[15] The pleura of one other animal was infiltrated to some extent with leukemia cells.

Cervical Region. The thyroid gland was examined in all but 2 of the leukemic guinea-pigs. Its stroma was infiltrated with leukemia cells. In animals showing the least degree of leukemia the infiltrating leukemia cells were found to the greatest extent in the perivascular tissue. The thyroid gland was almost completely replaced with leukemia cells in 3 of the leukemic guinea-pigs (Fig. 3). The thymus was replaced in 2 animals. The esophagus was normal in every case. The submucosa of the trachea was infiltrated with leukemia cells in several animals. In one, the tracheal wall was replaced by leukemia cells extending from leukemic tissue of the adjacent lymph nodes. The cervical lymph nodes were enlarged, and entirely or partially replaced by leukemia cells in all animals (Fig. 4). In lymph nodes with fat replacement atrophy the adipose tissue in the medulla and cortex was infiltrated with leukemia cells. The salivary glands showed infiltration with leukemia cells that extended from the periductal connective tissues into the glandular parenchyma.

Abdomen. The adipose tissue of the omentum was moderately infiltrated with leukemia cells. The lymph nodes of the omentum and of the entire abdominal area were replaced by leukemia cells in all of the leukemic guinea-pigs. Some lymph nodes were only slightly enlarged while others were massive with infiltration into adjacent tissue. In every animal, the spleen was enlarged and contained leukemia cells (Fig. 5). In less advanced cases, the white pulp was replaced by leukemia cells while the red pulp was only slightly infiltrated with

them. In advanced cases, splenomegaly was so great that the entire left half of the peritoneal cavity was occupied by the leukemic spleen. In these cases the normal architecture was not discernible. The stomach and intestine were normal except for replacement of lymphatic tissue, including Peyer's patches, by leukemia cells. The pancreas was only rarely involved, but, like other glandular organs infiltrated with leukemia cells, the tissue around its major ducts contained the abnormal cells. Enlargement of the liver was seen in all animals. Leukemia cells were found in the portal triads (Fig. 6), and in more advanced cases the sinusoids contained leukemia cells. Areas of necrosis and hemorrhage were found in the larger livers. Fatty degenerative changes were seen to a variable extent in hepatic cord cells, depending on the degree of leukemic infiltration. Glisson's capsule was infiltrated with leukemia cells in the advanced cases.

The gallbladder in one of the 10 leukemic animals was infiltrated with leukemic cells throughout its mucosa and muscular wall. In another guinea-pig only slight perivascular infiltration with leukemia cells was present in the gallbladder wall. In the remaining animals, this organ was not available for study. The medulla of the adrenal glands in all but one of the leukemic guinea-pigs was infiltrated with leukemia cells. In advanced cases the medulla was replaced and leukemia cells filled the vessels of the cortex. Two animals showed unilateral hyperplasia of the adrenal cortex.

The kidneys revealed masses of leukemia cells beneath the mucosa of the pelvis. This was a characteristic finding in all cases (Fig. 7). In advanced cases the peripelvic adipose tissue was widely infiltrated. The cortex of the kidney was generally free of leukemia cells except in advanced cases when capillaries of the cortex were filled with leukemia cells. A special type of leukemic infiltration was observed in the kidneys of a few animals. In these, the leukemia cells had a wedge-shaped or linear distribution extending from the cortex into the medulla (Figs. 7 and 8). This pattern is the same as that of the pyelonephritic scars seen in guinea-pigs. The ureters resembled the pelvis of the kidney in showing leukemia cells in the submucosa. The testes were normal. The epididymis of one was moderately infiltrated with leukemia cells. The submucosa of the urinary bladder of 2 animals contained many leukemia cells. One of these animals showed evidence of chronic cystitis. The hilum of the ovary of 3 animals was infiltrated with leukemia cells. The perivascular tissue of the myometrium of 7 guinea-pigs showed slight to marked infiltration.

Special Regional Examinations. The bone marrow revealed slight to marked leukemia in all but one animal. In this one exception no

definite leukemia cells were observed. Both femoral and sternal bone marrow were examined in each animal. The peripheral lymph nodes, including inguinal, axillary, and cervical, showed enlargement and partial or total replacement with leukemic tissue in every case. In the very large lymph nodes of one animal central areas of necrosis were observed. The skin of the 2 animals in which skin specimens were taken possessed no leukemia cells. The mammary gland of one female guinea-pig showed infiltration of leukemia cells around its major ducts. The eye was taken in one animal, and marked infiltration of leukemia cells in the choroid coat was found. Slight periductal infiltration of the lacrymal gland and perivascular infiltration of the periorbital adipose tissue also were observed.

Transplantable Leukemia

Leukemic tissue of 5 of the guinea-pigs with primary leukemia was transplanted to young inbred guinea-pigs of the same strain. One of the leukemias (no. 10) has only recently been transplanted. The other four transplants grew and killed the host animals. Table IV gives the general biologic characteristics of these transplantable leukemias.

Except for the tumor formed at the transplant site, the morphologic features of the transplantable leukemia resemble those of the primary cases. Leukemia L_2B has been studied at necropsy in animals killed at 8, 9, 11, 12, 13, 14, 15, 17, 18, and 21 days after subcutaneous transplantation of pieces of leukemic spleen from an animal of the ninth transplant generation. One guinea-pig was killed on each of the days listed. Peripheral blood counts were made beginning the day following transplantation and at short intervals thereafter until all guinea-pigs had been killed and necropsied. Text-figure 3 shows the leukocyte counts. Depression of the leukocytes occurred on the ninth day after transplantation, as compared with the first day. At the time of the low peripheral leukocyte count, replacement of splenic follicles with leukemic tissue was a striking anatomical finding.

The necropsy material of the leukemic animal at 8 days after transplantation revealed leukemia cells at the site of the transplant widely infiltrating along tissue planes of the body wall. It also showed dissemination of leukemia cells to regional lymph nodes and the white pulp of the spleen. At 9 days, all lymph nodes were infiltrated with leukemia cells. On the eleventh day the spleen was enlarged and the red and white pulp were strikingly infiltrated with leukemia cells. Bone marrow, thymus, lung, liver, and medulla of the adrenal gland showed infiltration with leukemia cells. The subsequent course

TABLE IV

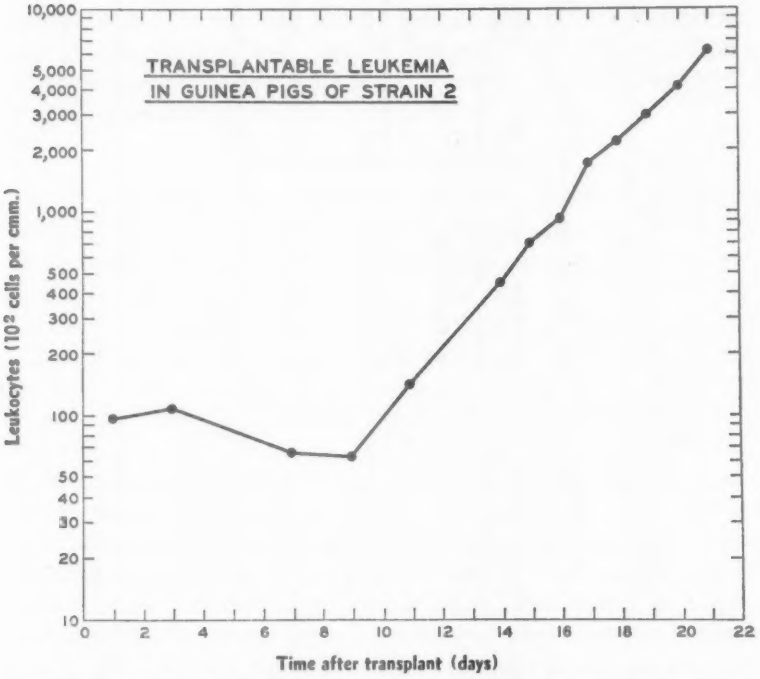
Transplantable Leukemias in Inbred Guinea-Pigs

Tumor designation and animal of origin	Highest leukocyte count observed	Tumor formation at transplant site	Latent period for appearance of leukemia	Interval from transplantation to death	Tissues transplanted	Routes of transplantation	Takes	Number of transplant generations
							%	
L2 1	843,600	Tumor appears before blood becomes leukemic; at death very large tumor	4 to 5 mos. in early generation, 1 to 2 mos. after 10th generation	Approximately 5 to 6 mos. in early generation, approximately 2 mos. in later generations	Tumor Spleen Lymph node Blood	Subcutaneous Intraperitoneal	100	17
L2B 8	960,000	Forms small congested, hemorrhagic area of infiltration	7 to 9 mos. for 1st generation, 1 mo. for 2nd generation, thereafter approximately 10 to 14 days	Approximately 20 days from 3rd generation to present	Spleen Lymph node Blood	Subcutaneous Intraperitoneal Intravenous	100	32
L2C 3	39,200	Forms small congested area of infiltration	3 to 5 mos. in 1st generation	Not yet determined	Spleen	Subcutaneous	100	2
L13 9	462,600	Formed large tumor mass	Approximately 6 wks. in 1st generation, 1 to 4½ mos. in subsequent generations	Approximately 2½ to 3½ mos.	Lymph node	Subcutaneous	100*	Lost after 4th transplant generation

* In one generation failure of the tumor to take was attributed to faulty transplantation technique.

of the disease revealed enlargement and complete replacement of all lymphatic tissue. The perivascular and periductal infiltration with leukemia cells in all organs and tissues increased tremendously. In terminal stages of the disease, all the blood vessels showed excessive numbers of leukemia cells. Figures 9, 10, 11, 12, and 13 show leukemic infiltration in several organs and tissues.

In transplantable leukemia of the guinea-pig, the development of



Text-fig. 3. Average total leukocyte counts taken periodically for a series of guinea-pigs receiving a transplantable leukemia. Leukemia line L2B.

a rapid rise in the leukocyte count indicated in most instances that death would follow in a few days. This was true of the L2B series in particular. The route of injection had no apparent influence on the course of the disease. The possible effects of size of transplant pieces or number of leukemia cells on the course of the disease was not determined.

Spontaneous regression was not observed. The localization of leukemia cells in chronic inflammatory lesions, as noted in the primary cases, was observed in the lung and kidney; but, since the host

animals were 3 to 4 months of age in most instances and did not often have chronic inflammatory processes, this finding was relatively less evident in the animals with transplanted tumors than in the cases of primary leukemia.

Discussion

The over-all incidence of leukemia in the present series of 303 guinea-pigs, 1 year of age or over, was 3.3 per cent. No significant difference was found in incidence between irradiated animals and the control group. However, the impression was gained that leukemia appeared at an earlier age in the irradiated animals than in the controls. In the series of 111 carcinogen-treated guinea-pigs reported on by Heston and Deringer[14] the incidence of lymphatic leukemia was 2.7 per cent. Combining the two series gave an incidence of leukemia of approximately 3 per cent in 414 guinea-pigs.

Since both chemical carcinogens and x-irradiation will induce lymphatic leukemia in some laboratory animals,[16,17] it seems unwarranted to conclude from the data available at the present time that these agents are non-cancerigenic in the guinea-pig, as far as leukemia is concerned.

Lymphatic leukemia and lymphosarcoma in guinea-pigs does not differ from that observed in other rodents.[18] A special organ susceptibility, such as that of the thymus for the origin of lymphatic tumors in some strains of mice,[19] has not been noted. However, the more marked leukemic involvement of lymph nodes and spleen in comparison with bone marrow, liver, and thymus in the less advanced cases reported here suggests origin from these sites.

In the transplantation series the survival time of the host and degree of local tumor formation of the leukemia showed some variation, and in general resembled the transplantable lymphatic leukemias observed in mice.[18]

Data on the leukocyte count throughout the life span of the guinea-pigs which developed leukemia were of interest because for most of the animals it indicated a sudden appearance of the disease without a preleukemic alteration in the peripheral blood. The 2 animals which showed prolonged variation in peripheral leukocyte counts before developing leukemia were of interest, but the lack of information on the histologic state of the lymphatic tissues during the preleukemic period limits the usefulness of this data.

The relation of leukemic infiltration to inflammatory lesions noted in several animals raised questions which can only be answered by additional experimental work with transmissible leukemia. Since

these inflammatory lesions usually contain lymphocytes or lymphatic tissue in varying amounts, the factors which determine the localization of leukemia cells in any normal lymphatic tissue may operate to produce localization in the inflammatory areas. The varied appearance of pulmonary involvement by malignant lymphoma that Robbins[20] described roentgenologically might possibly be explained on an inflammatory basis. The action of a *locus minoris resistentiae* might also be considered in explaining the localization of leukemia cells in the inflammatory areas. In virus tumor induction studies, the operation of a *locus minoris resistentiae* is well known.[21]

Conclusions

Lymphatic leukemia occurred in 3.6 per cent of 112 strain 2 guinea-pigs, in 6.7 per cent of 30 strain 13 guinea-pigs, and in 2.5 per cent of 161 heterogeneous hybrid guinea-pigs.

X-irradiation and gamma irradiation have not been established as leukemogenic agents for the guinea-pig.

In 6 guinea-pigs leukemia appeared suddenly without evidence of preceding alterations of the peripheral leukocyte count. In 2 animals the peripheral leukocyte count was altered for prolonged periods prior to the appearance of leukemia.

At necropsy the 10 animals with primary lymphatic leukemia were remarkably alike.

Several animals showed localization of leukemia cells at old inflammatory sites in the lungs, kidneys, and urinary bladder.

Transplantation of leukemia was accomplished for 4 of the primary cases. It was attempted only in inbred strains and 100 per cent takes were observed.

The different transplant lines showed variation in the duration of the disease and degree of local tumor formation.

References

1. Lorenz, E., Jacobson, L. O., Heston, W. E., Eschenbrenner, A. B., Shimkin, M., Deringer, M., Doniger, J., and Schweisthal, R. Biological Effect of Long-Continued Total Body Gamma Irradiation of Mice, Guinea Pigs and Rabbits. Part III, Vol. 22B, National Nuclear Energy Series, Div. IV, McGraw-Hill Co., New York. (In press.)
2. Lorenz, E., Jacobson, L. O., Uphoff, D., Sutton, H., and Schweisthal, R. The Blood Picture of Acutely Exposed Hybrid Guinea Pigs. Quarterly Report, Argonne National Laboratory, Division of Biological and Medical Research. Aug., Sept., Oct., 1950, ANL-4531, pp. 27–39.
3. Dickson, E. C. Sarcoma occurring in a guinea-pig. *Proc. Soc. Exper. Biol. & Med.*, 1915–16, 13, 26–27.

4. Miguez, C. Sarcoma espontáneo transplantable en el cobayo. *Rev. d. Inst. bact., Buenos Aires*, 1918, **1**, 147–154.
5. Fischer, G., and Kantor, L. Contribución al estudio del parentesco entre especies animales por métodos biológicos. *Rev. d. Inst. bact., Buenos Aires*, 1919, **2**, 303–308.
6. Bender, L. Sarcoma of the heart in a guinea pig. *J. Cancer Research*, 1925, **9**, 384–387.
7. Snijders, E. P. Over een overentbare leukaemie bij cavia's. *Nederl. tijdschr. v. geneesk.*, 1926, **70**, 1256–1262.
8. Snijders, E. P. Eenige resultaten van proefnemingen mit een overentbare cavia leukose. Handl. XXI. *Nederl. Natuur- en Geneesk. Congr., Amsterdam*, 1927, pp. 160–164.
9. Tio Tjwan Gie. Over leukaemie bij dieren en over een overentbare cavia-leukose. Inaugural Dissertation. Amsterdam, 1927, 192 pp.
10. de Bruyn, W. Personal communication, 1952.
11. Gerlach, F. Die praktisch wichtigen Spontaninfektionen der Versuchstiere. In: Kolle, W., Kraus, R., and Uhlenhuth, P. (ed.) Handbuch der pathogenen Mikroorganismen. G. Fischer, Jena, 1929, **9**, 521–522.
12. Ball, V., and Pagnon, F. Sarcome à cellules rondes du rein chez un cobaye. *Bull. Soc. Sc. Vét. Lyon*, 1935, **38**, 49–51.
13. Rogers, J. B. Tumors of guinea pigs. (Abstract.) *Anat. Rec.*, 1951, **109**, 343.
14. Heston, W. E., and Deringer, M. K. Induction of pulmonary tumors in guinea pigs by intravenous injection of methylcholanthrene and dibenzanthracene. *J. Nat. Cancer Inst.*, 1952, **13**, 705–717.
15. Congdon, C. C., Uphoff, D., and Lorenz, E. Modification of acute irradiation injury in mice and guinea pigs by injection of bone marrow: A histopathologic study. *J. Nat. Cancer Inst.*, 1952, **13**, 73–107.
16. Shay, H., Gruenstein, M., Marx, H. E., and Glazer, L. The development of lymphatic and myelogenous leukemia in Wistar rats following gastric instillation of methylcholanthrene. *Cancer Research*, 1951, **11**, 29–34.
17. Kirschbaum, A. Rodent leukemia: Recent biological studies. A review. *Cancer Research*, 1951, **11**, 741–752.
18. Engelbreth-Holm, J. Spontaneous and Experimental Leukaemia in Animals. Oliver & Boyd, London, 1942, pp. 40–52.
19. Kaplan, H. S. Comparative susceptibility of the lymphoid tissues of strain C57 black mice to the induction of lymphoid tumors by irradiation. *J. Nat. Cancer Inst.*, 1947–48, **8**, 191–197.
20. Robbins, L. L. The roentgenological appearance of parenchymal involvement of the lung by malignant lymphoma. *Cancer*, 1953, **6**, 80–88.
21. Rous, P., Murphy, J. B., and Tytler, W. H. The rôle of injury in the production of a chicken sarcoma by a filterable agent. *J. A. M. A.*, 1912, **58**, 1751.

[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Lymphatic leukemia cells in the peripheral blood of case 7. × 1260.

FIG. 2. Case 2. Leukemia cells replacing a heterotopic lymph nodule in an old inflammatory focus in the lung. × 270.

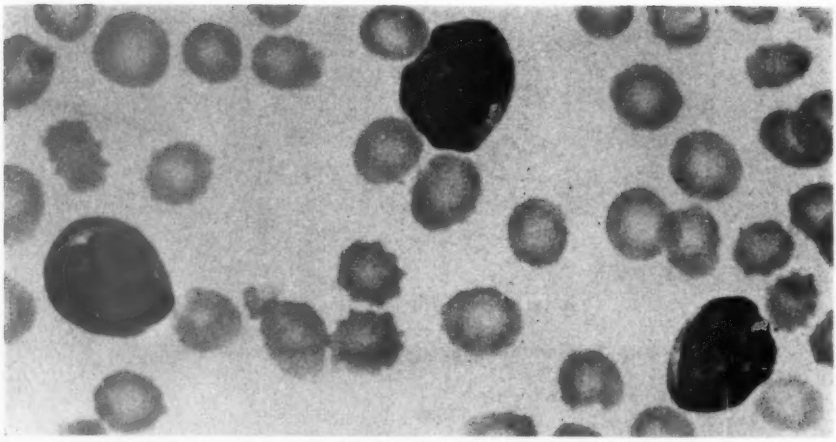
FIG. 3. Case 2. Leukemic replacement of thyroid gland parenchyma. × 350.

1

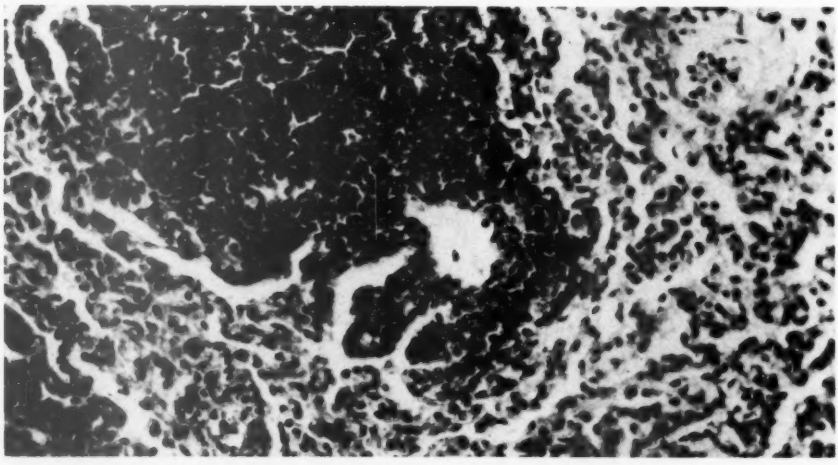
2

3

1



2



3

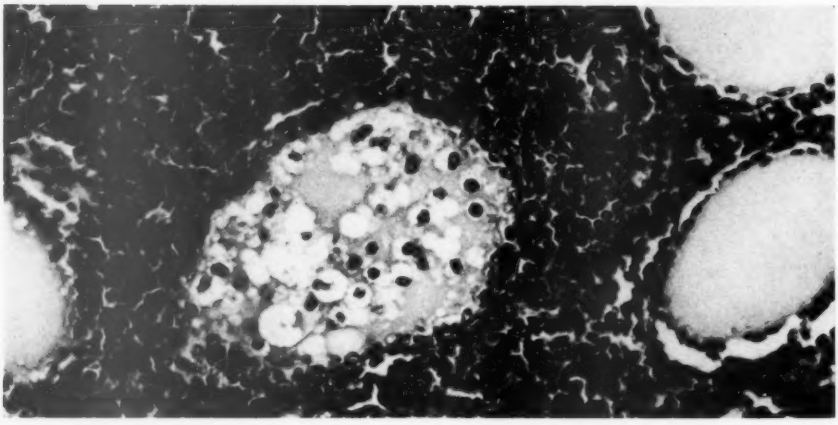


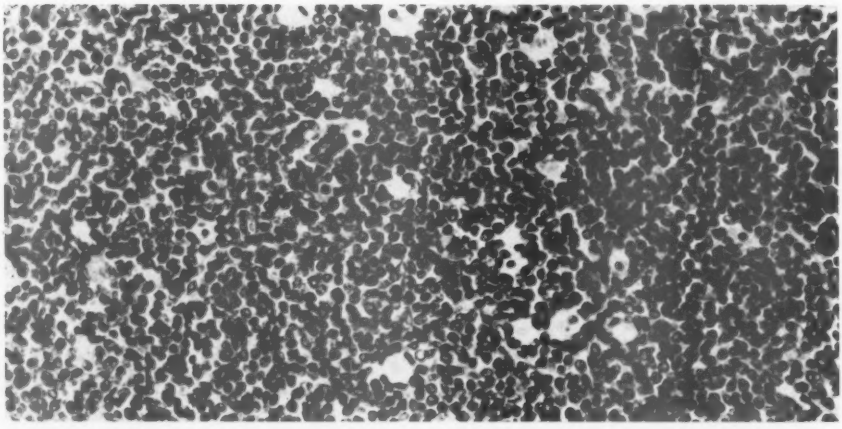
FIG. 4. Case 1. Leukemia cells in the cortex of a lymph node. × 350.

FIG. 5. Case 2. Leukemic spleen. Groups of erythropoietic cells are present in sinusoids near the right margin of the photograph. × 350.

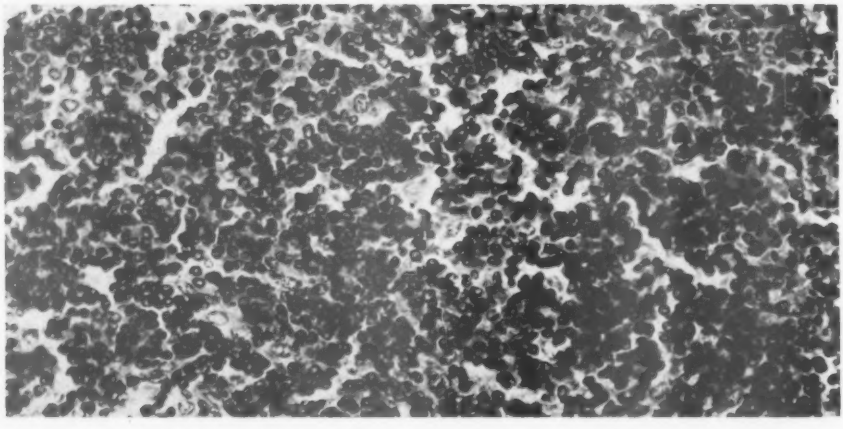
FIG. 6. Case 2. Leukemic infiltration in the portal region of the liver. Numerous leukemia cells in sinusoids. × 350.

FIG. 7. Case 6. Cross section of a guinea-pig kidney showing the usual leukemic infiltration beneath the pelvic mucosa and a wedge-shaped area of leukemic infiltration extending from the cortex into the medulla. × 8.

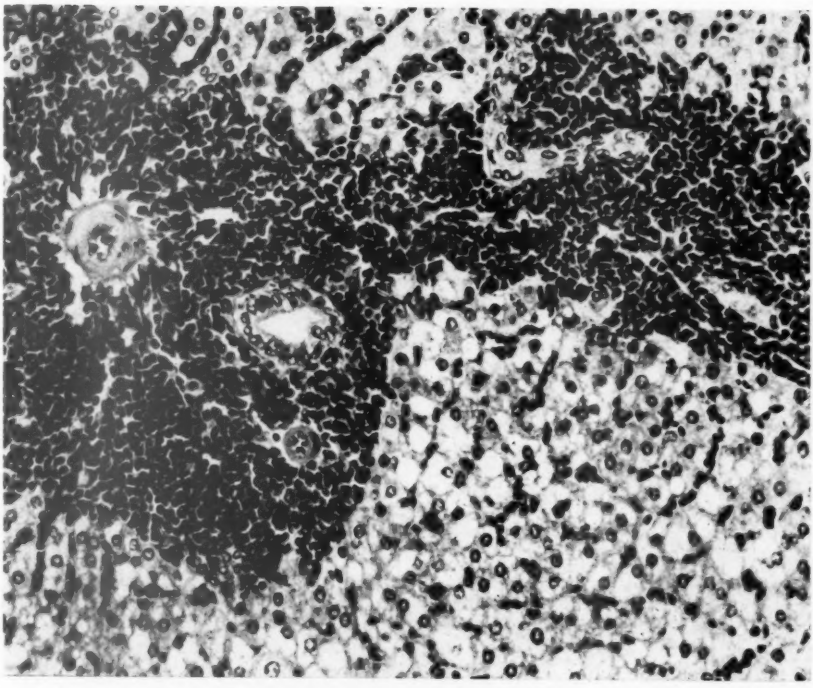
4



5



6



7

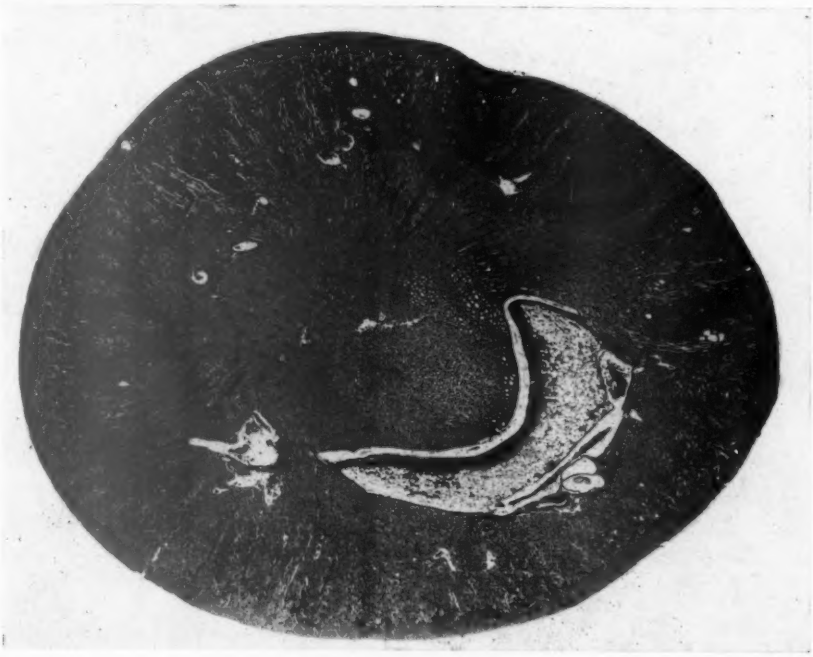
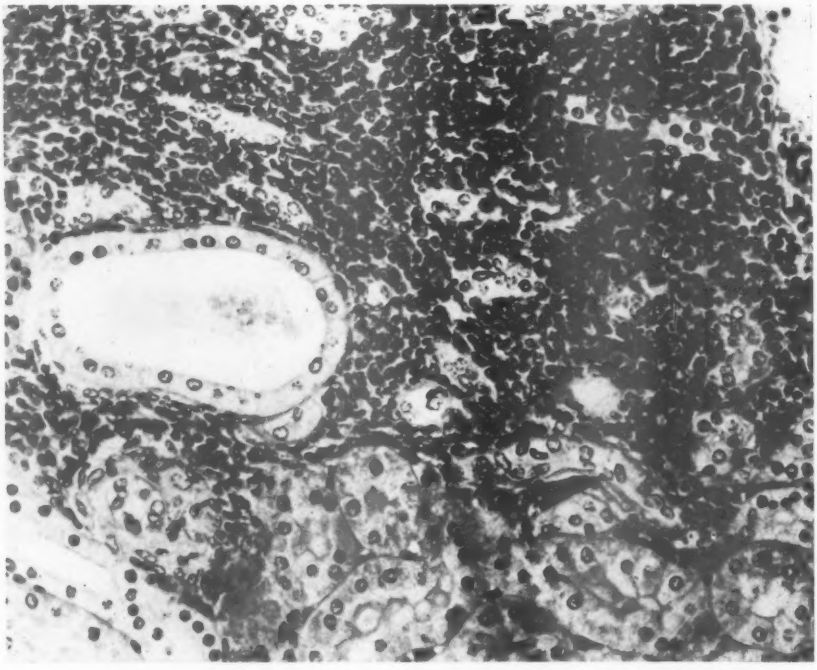


FIG. 8. Case 6. High-power view of the edge of the pyelonephritic scar shown in Figure 7. × 350.

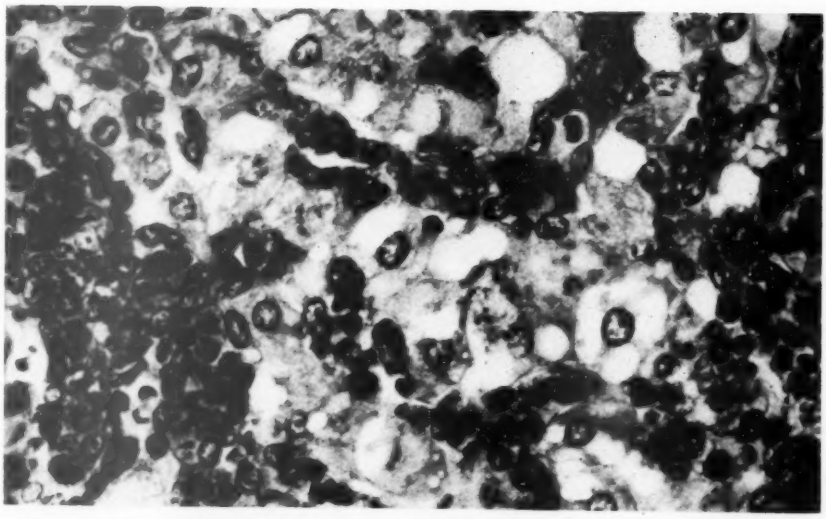
FIG. 9. Leukemic infiltration in the liver of an animal from the sixth transplant generation of line L_2B. × 570.

FIG. 10. Leukemic infiltration of the femoral bone marrow of an animal from the fourth transplant generation of line L_2B. × 630.

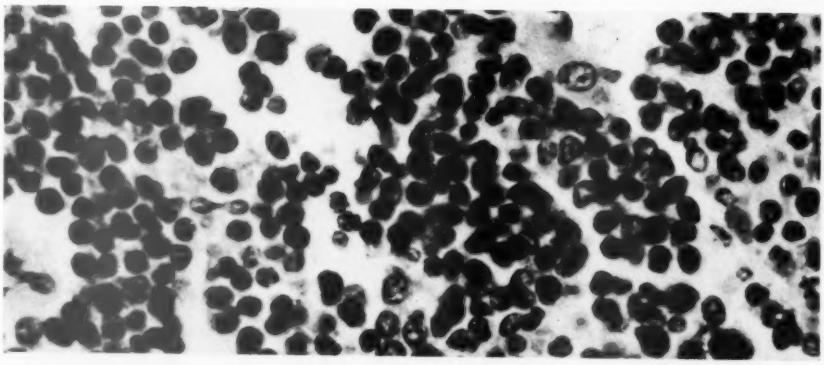
FIG. 11. Leukemic infiltration in the medulla of the adrenal gland of an animal from the sixth transplant generation of line L_2B. × 440.



9



10



11

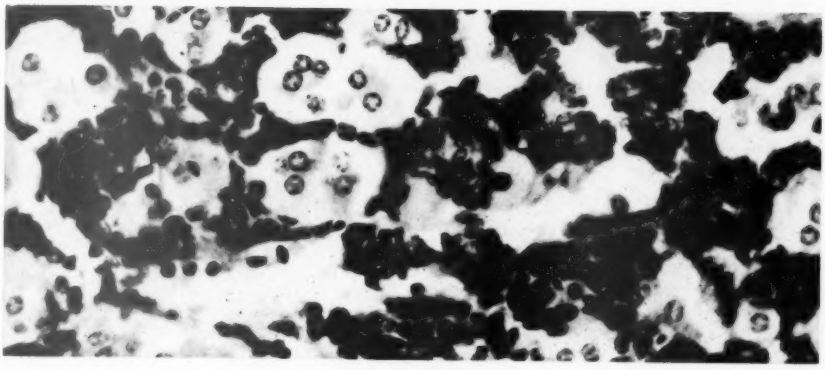
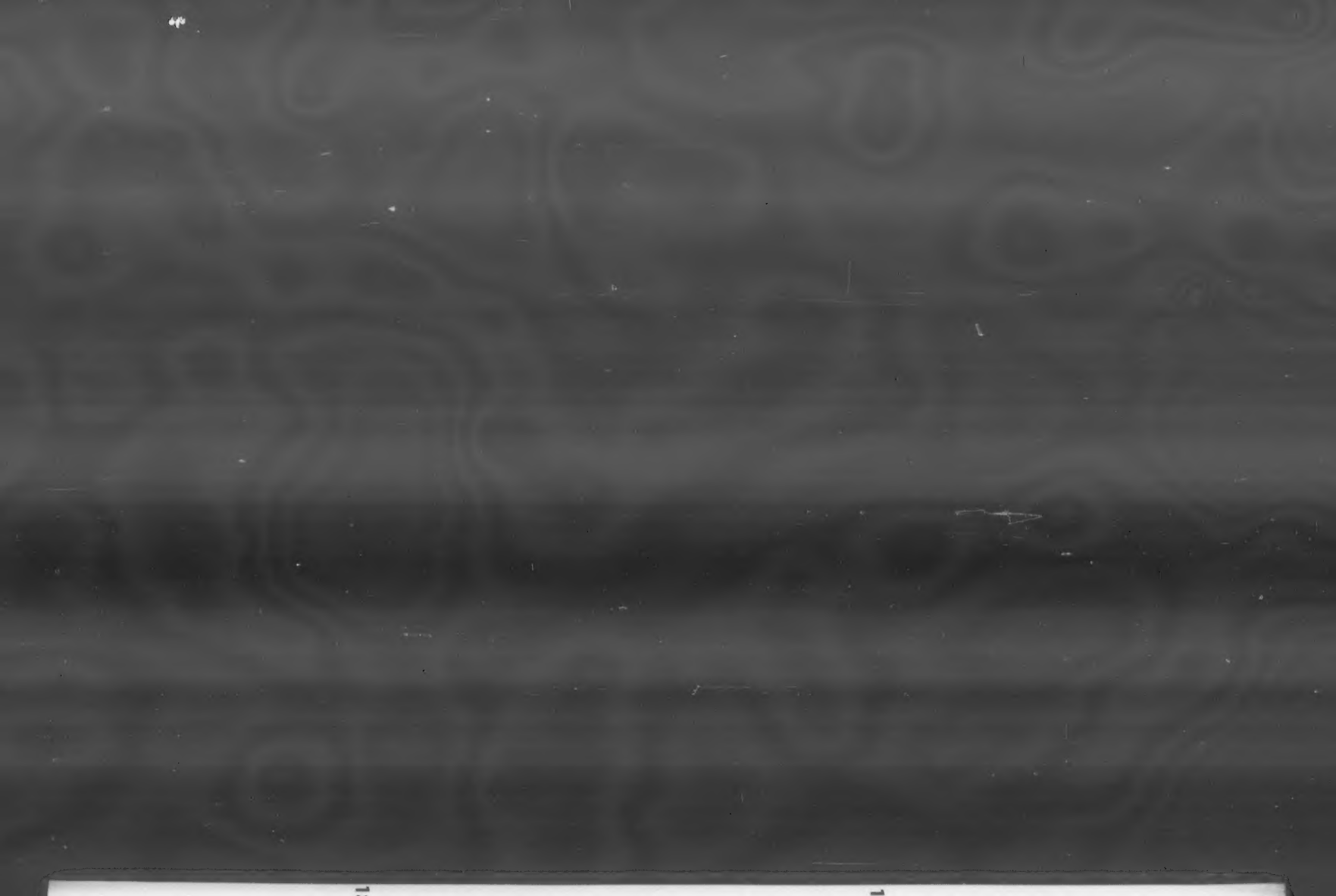
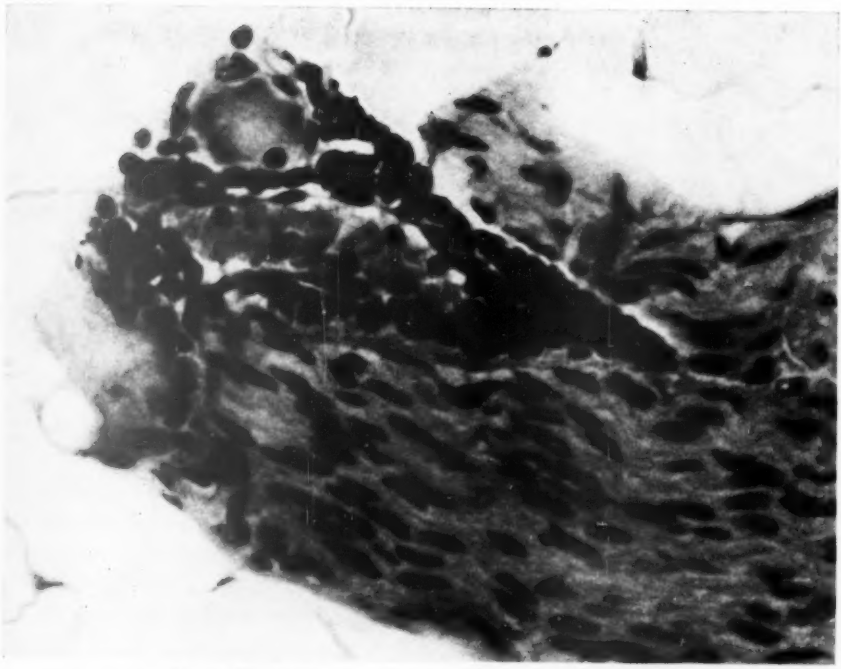


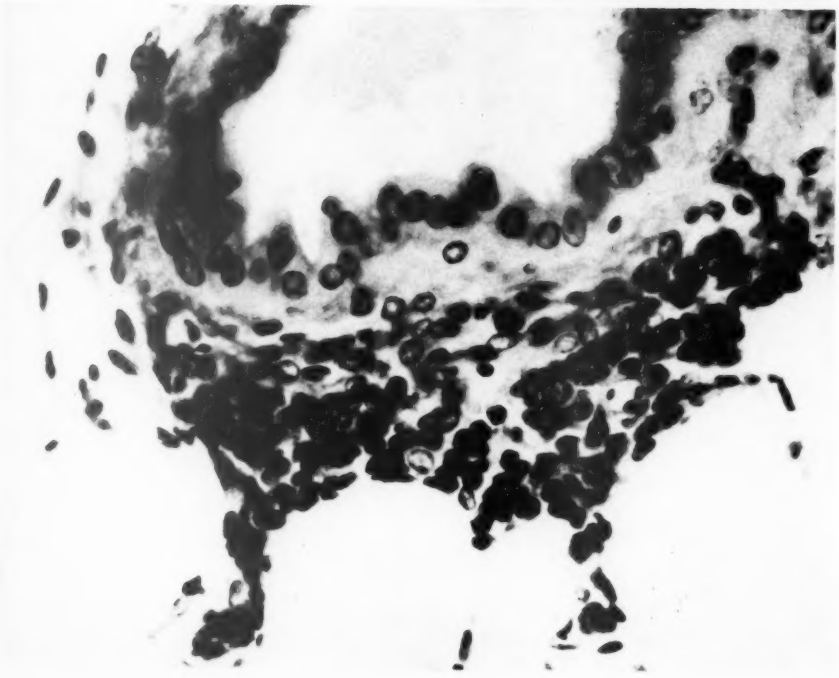
FIG. 12. Perivascular leukemic infiltration in a sympathetic ganglion near the ureter from an animal of the tenth transplant generation of line L_2B. × 500.

FIG. 13. Periductal leukemic infiltration in the pancreas of an animal of the sixth transplant generation of line L_2B. × 555.





12



13

WIDESPREAD ACUTE NECROSIS OF THE LIVER FOLLOWING SULFONAMIDE THERAPY IN PATIENTS WITH LEUKEMIA *

K. V. Lodge, M.B., Ch.B., and A. S. Woodcock, M.B., Ch.B.
(From the Department of Pathology, University of Manchester, Manchester 13, England)

Hepatic necrosis, either focal or massive, is a well recognized though infrequent complication of sulfonamide therapy. Massive necrosis confirmed by post-mortem examination has been reported by Cline[1] (1938), Russell[2] (1940), Berger and Applebaum[3] (1941), Herbut and Scaricaciottoli[4] (1945, 2 cases), More, McMillan, and Duff[5] (1946, 2 cases), and Krusius[6] (1947, 2 cases). In none of these was leukemia also present, nor in several additional cases diagnosed clinically, but in which necropsies were not performed.

We have recently noted extensive acute hepatic necrosis at necropsy on 3 patients with leukemia. On inquiry we found that shortly before death all 3 had received sulfonamides, a fact which we believed played a part in the production of the necrosis.

We have been unable to find any report of hepatic necrosis as a complication of leukemia itself. Since leukemic patients are not unlikely to receive sulfonamides in the course of their disease, either before the diagnosis is established, or as advocated by Merrill[7] (1948) for the control of intercurrent infections, it seemed desirable to investigate the possible inter-relationship of hepatic necrosis, leukemia, and sulfonamide therapy. We therefore reviewed 97 unselected cases of leukemia in which necropsies had been performed, and inquired whether the patients had received sulfonamides. As anoxia appeared a possible cause of hepatic necrosis, the patients' terminal hemoglobin levels were noted, and liver sections were studied histologically to determine the degree of chronic passive congestion and the extent of leukemic infiltration.

Case Reports of Patients with Liver Necrosis

Case 1

An 8-year-old girl of previously good medical history became "out of sorts" in August, 1950. Her parents attributed this to a fall from her bicycle in which she sustained a cut chin but no other injury. In late September she was feverish and developed a sore throat for which 1 gm. of sulfamezathine every 4 hours was prescribed. A course of 9 gm. was given, and the patient became apyrexial and symptomless. Five days later she again became feverish, and two injections of penicillin, each of 30,000 units, were given. There was no improvement, and a second course of sulfa-

* Received for publication, August 6, 1953.

mezathine was started at the original dosage of 1 gm. every 4 hours. Vomiting commenced, and the drug was stopped when the second course had totalled 9 gm. The child's condition deteriorated, and she was hospitalized on October 16. On admission she appeared ill, pale, and slightly jaundiced. The temperature was 100.5°F., and the pulse rate 100 per minute. Both tonsils were irregularly enlarged, but not ulcerated. There were enlarged glands in axillae and groins, and the liver and spleen were palpable. A blood count on the day after admission showed: red cells, 3,800,000 per cmm.; hemoglobin, 10.1 gm. per 100 ml. (68 per cent, Haldane); white cells, 8,000 per cmm.; polymorphonuclear cells, 3.5 per cent; lymphocytes and lymphoblasts, 93 per cent, large mononuclear cells, 3.5 per cent, eosinophils, 0 per cent, basophils, 0 per cent. Platelets were scanty. Biopsy of the vertebral bone marrow showed the typical appearances of acute lymphatic leukemia.

The patient became increasingly jaundiced, and was delirious with screaming attacks, finally lapsing into coma and dying on October 18, 1950, 2 days after admission. No drugs were given in the hospital apart from 40 mg. of ACTH 3 hours before death.

Relevant Post-Mortem Findings

The body was that of a well nourished child of average development. There was moderate icterus of skin and conjunctivae. The normal site of the thymus was occupied by an irregular mass of firm, pinkish white tissue measuring 10 by 10 by 6 cm. Thoracic and abdominal lymph nodes were enlarged up to 2 cm. in diameter and were firm and gray on section. The liver was slightly enlarged, weighing 830 gm. It was soft and the capsule was smooth. The cut surface showed an irregular, coarse, red and yellow mottling in which no lobular pattern could be defined. The extrahepatic biliary channels were normally patent. The kidneys were enlarged, the right weighing 130 gm. and the left, 135 gm., with smooth surfaces. On section there was pallor of both cortex and medulla, but no other macroscopic abnormality.

Histologic Appearances. The lymph nodes, spleen, thymic mass, and tonsils showed loss of normal architecture, with infiltration by cells of the lymphocyte series. The appearances were those of lymphatic leukemia.

The liver showed diffuse necrosis involving almost its whole substance (Fig. 1), although the changes appeared most marked centrilobularly. Surviving liver cells were swollen and globular (Fig. 2), some with intensely eosinophilic cytoplasm, others containing large vacuoles, which were shown to contain fat in frozen sections stained with Sudan IV (Fig. 3). There was loss of nuclei in many cells, the remainder showing varying degrees of karyolysis. The liver architecture was maintained by the reticulum of the sinusoids which were dilated and congested. The few inflammatory cells were predominantly mononuclear, with occasional polymorphonuclear leukocytes. The portal tracts showed very slight bile duct proliferation, but no bile

plugs were seen. There was moderate leukemic infiltration confined to the portal tracts and the outermost third of the lobules.

The kidney showed normal glomeruli. The cytoplasm of the cells of the proximal convoluted tubules was foamy, with vacuoles usually situated near the cell base. Similar but less marked changes were seen in the distal convoluted and collecting tubules. Frozen sections stained with Sudan IV showed large quantities of fat in the cells of the proximal convoluted tubules (Fig. 4), and smaller amounts throughout the remainder of the tubular system. No sulfonamide precipitation was seen. There was no inflammatory cell or leukemic infiltration of the kidney, and there were no vascular lesions.

Case 2

A man, 24 years of age, was admitted to the hospital, having had a boil on the back of his neck for 10 days. A course of sulfathiazole, 1 gm. every 4 hours, had been given in the week prior to admission. On examination he was pale. His temperature was 101°F. and his pulse rate 96 per minute. There was enlargement of the cervical lymph nodes and the spleen was palpable 5 cm. below the costal margin. The blood count on admission showed: red cells, 3,470,000 per cmm.; hemoglobin, 9.45 gm. per 100 ml. (61 per cent, Haldane); white cells, 1,700 per cmm., with 51 per cent polymorphonuclear cells, 37 per cent lymphocytes, 4 per cent monocytes, 8 per cent metamyelocytes; platelets were scanty. Repeated blood cultures failed to yield growth of organisms on aerobic and micro-aerophilic incubation. The bone marrow of the sternum and iliac crest showed a marked reduction in the number of cells of the erythroid and myeloid series; 85 per cent of the cells present were lymphocytes or their precursors. The appearances were those of lymphatic leukemia. After admission the hemoglobin and red cell count fell steadily in spite of repeated transfusions and there was rapid enlargement of the spleen and liver. The patient's general condition showed gradual deterioration until 2 months after admission when he developed sepsis of the skin over the ankle at the site of a transfusion incision and a compress of penicillin and sulfathiazole was applied. Following this his condition deteriorated rapidly; he became mentally disorientated and died 3 days later.

Relevant Post-Mortem Findings

The body was that of a well nourished young man with an icteric tinge of the skin and conjunctivae. There were petechial hemorrhages over the arms, legs, and trunk. The superficial lymph nodes in the cervical, axillary, and inguinal regions were enlarged. The liver was grossly enlarged, weighing 3,480 gm., and had a smooth surface. The cut surface was mottled by many small areas of white tissue scattered diffusely through the liver substance, and there were other larger areas into which there had been hemorrhage. The spleen was considerably enlarged, weighing 1,780 gm. On section the pulp was light red and very firm; there were several darker red areas which resembled infarcts. The kidneys were enlarged, weighing 300 gm. each; their capsules stripped easily leaving a smooth surface. On section there

were numerous deposits of white tissue up to 0.5 cm. in diameter throughout the cortex. The mesenteric, para-aortic, and hilar lymph nodes were enlarged. The femur on section showed many islands of red marrow.

Histologic Appearances. The liver showed extensive areas of necrosis with complete destruction of liver cells; each of these necrotic areas involved several lobules and there was considerable hemorrhage into them, with moderate polymorphonuclear leukocytic infiltration (Figs. 5 and 6). There were small areas of leukemic infiltration, chiefly of centrilobular distribution, and in these areas the cells were all lymphocytes. The kidneys contained scattered foci of leukemic infiltration in both cortex and medulla. The glomeruli appeared normal and the epithelium of the tubules was not vacuolated. No vascular lesions were seen. The spleen and lymph nodes showed loss of follicular pattern with diffuse infiltration by lymphocytes. There were some infarcts with surrounding hemorrhage in the spleen. The marrow of the femur showed active hemopoiesis and large numbers of lymphoblasts and lymphocytes were present. The appearances of the spleen, lymph nodes, and bone marrow were typically those of lymphatic leukemia.

Case 3

A man, 75 years old, was admitted to the hospital with a history of dyspnea on exertion. The dyspnea had been present for 12 months but had become more severe during the 6 weeks prior to admission. During that time he had also developed cough with sputum. On examination the temperature was 101° F.; pulse, 88 per minute; blood pressure, 135/90 mm. of Hg. He was moderately dyspneic at rest, very pale, but not jaundiced, and had slight edema of both ankles. There was poor expansion of the chest with widespread crepitation over both lung fields. The liver and spleen were not felt and the superficial lymph nodes were not enlarged. The patient was considered to be suffering from chronic bronchitis with heart failure and was treated with 25,000 units of penicillin every 6 hours. No other drugs were given. Roentgenograms of the chest showed a mass in the mediastinum.

The patient's condition rapidly deteriorated. He became disorientated and died 5 days after admission.

Subsequent inquiry from the general practitioner revealed that the patient had received a course of sulfonamide immediately prior to admission. The total amount taken was not known but there was no reason to believe that it was excessive.

Relevant Post-Mortem Findings

The body was that of an obese, elderly man. The lymph nodes in the cervical, axillary, and inguinal regions were not enlarged. The pleura of the right lung was thickened and adherent to the chest wall. Both lungs showed considerable congestion and edema of the lower lobes. The bronchi and bronchioles contained mucopurulent exudate

and the lining mucosa was congested and roughened. There was a mass at the hilum of the right lung, consisting of numerous lymph nodes enlarged up to 3 cm. in diameter. On section the glands were pink and homogeneous. The slightly enlarged liver weighed 1,840 gm., and had a smooth surface. On section the parenchyma was pale but the lobular pattern was maintained. Numerous white nodules, varying in size up to 0.5 cm. in diameter, were scattered through the liver tissue. The spleen was enlarged, weighing 370 gm. On section the pulp was dark red. The kidneys were of normal size; their capsules stripped easily leaving a smooth surface. On section, the cortex, medulla, and pelves showed no abnormality. The bone marrow was red and hyperplastic.

Histologic Appearances. Sections of the liver showed numerous focal areas of necrosis (Figs. 7 and 8) which appeared to correspond to the white nodules described macroscopically. These areas were centrilobular in distribution but in many places coalesced to involve several lobules. In the areas of necrosis there was polymorphonuclear leukocytic infiltration. The remainder of the liver showed very slight passive congestion. Leukemic infiltration, which was of lymphocytic type, was moderate and confined to the periportal areas; everywhere it was well demarcated from the areas of necrosis. The spleen and lymph nodes showed loss of follicular pattern and diffuse infiltration by lymphocytes. The femoral bone marrow showed active erythropoiesis and widespread infiltration by cells of the lymphocyte series. The appearances were typically those of lymphatic leukemia. The kidney showed no abnormality of glomeruli, tubules, or blood vessels, and there was no leukemic infiltration.

In sections of the livers from all 3 cases stained by the Gram technique no organisms were seen.

Discussion

In cases 1 and 2 the hepatic necrosis was massive, and both appeared clinically to terminate in liver failure. In case 3 the necrosis was not so extensive.

Before ascribing the liver damage to sulfonamide, other possible causes were considered. Other drugs were excluded. The first patient (case 1) received only two doses of penicillin, which is not known to be hepatotoxic, and a dose of ACTH given within 3 hours of death. The second patient (case 2) received penicillin, liver extracts, iron, and vitamins. In case 3 only penicillin was administered. None of these agents nor the blood transfusions given in case 2, seemed related to

the hepatic necrosis. The possibility of necrosis due to leukemic infiltration was considered. In none of the 3 cases was infiltration marked, nor were areas of necrosis in constant association with areas of infiltration. Moreover, of the 97 additional cases of leukemia reviewed, there were 27 which showed massive leukemic infiltration, but none of these showed any necrosis.

The changes of chronic passive congestion were very slight in all 3 cases. Furthermore, in the whole series reviewed, of 24 cases with marked passive congestion of the liver histologically, of 17 with evidence of severe anemia (hemoglobin below 4.44 gm. per 100 ml. or 30 per cent, Haldane), and of 7 in which there was a combination of these features, no case showed areas of hepatic necrosis.

The possibility that the hepatic lesions were due to bacterial infection was considered. Only in case 2 was there a history compatible with septicemia, but repeated blood cultures from this patient were negative. In all 3 cases there was no necrosis or abscess formation in any other organs at necropsy, and no bacteria were seen on Gram staining of liver sections. We believe, therefore, that the sulfonamides were responsible for the hepatic necrosis.

MECHANISM OF PRODUCTION OF NECROSIS

Of the mechanisms by which sulfonamide might produce hepatic necrosis, allergy rather than a direct chemical toxic action has been stressed by previous writers. Multiple foci of necrosis in the liver and other organs have been described, as by Lederer and Rosenblatt[8] (1942), and ascribed to allergy. The vascular lesions of polyarteritis nodosa subsequent to sulfonamide therapy are generally accepted as allergic in origin, but in our cases there were no vascular lesions, and damage was confined to the liver, except in case 1 in which there was marked fatty change throughout the renal tubules, but no sulfonamide precipitation, and no necrosis or inflammatory cell infiltration of the renal parenchyma. The "nephrosis of undetermined etiology" which Cline[1] (1938) mentioned in his report, the tubular degeneration without sulfonamide crystal formation described by Herbut and Scaricaciottoli[4] (1945), and the changes in the kidney described by Krusius[6] (1947) seem similar to the appearances in our case. We agree with Herbut that these changes might be regarded as secondary to the liver damage and not directly due to the sulfonamide.

In favor of the theory of allergy is the fact that in only 2 previously reported cases (Cline,[1] 1938; Russell,[2] 1940), did the patients receive

excessive amounts of sulfonamide. The significance of two courses of the drug, with an interval in which sensitization might occur, has been stressed by Herbut and Scaricaciottoli[4] (1945). Rammelkamp[9] (1948), in a study of the toxic effects of the sulfonamides, noted jaundice developing 2 to 7 days after commencement of a second course, a pattern to which our first case conforms. It is known that a hyperreactive state to sulfonamides may persist for many months (Erskine,[10] 1939), and in addition to our first case, 5 of those previously reported (Cline[1]; Berger and Applebaum[3]; Herbut and Scaricaciottoli,[4] case 1; More *et al.*,[5] both cases), in which two courses of drug were given, might be explained on the basis of acquired sensitivity. Our second case is of particular interest in that, although only one course of the drug was given orally, sulfonamide powder was applied locally to an intravenous drip wound 3 days before death. Absorption from this site would be slight and a hypersensitivity reaction rather than direct toxicity would therefore appear to be the probable mechanism for liver damage. In our third case and in the other 4 previously reported cases to which we have referred, only one course of the drug is known to have been given. These might be explained on the basis of anaphylactic hypersensitivity in a manner similar to that postulated by Rich[11] (1942) in his discussion of vascular lesions following sulfonamide therapy.

Focal but not diffuse hepatic necrosis can be produced in animals by very high dosage of sulfonamides (Rake, van Dyke, and Corwin,[12] 1940), and on this evidence Herbut and Scaricaciottoli[4] (1945) suggested that focal hepatic necrosis in man may be of direct toxic origin, but that the diffuse form is due to sensitization. It seems more reasonable to suggest, as do More *et al.*[5] (1946), that both forms are of similar allergic origin, and that the diffuse form is a progression from the focal, as is illustrated by the histologic appearances in our third case.

Direct patch and intradermal tests with the drug for sulfonamide sensitivity are unreliable. Leftwich[13] (1944) evolved a method based on the hypothesis that sulfonamides are capable of antigenic activity only when attached to plasma proteins. He used the serum of patients receiving homologous sulfonamide to produce positive intradermal reactions in a group of patients with toxic symptoms clinically considered due to sulfonamide sensitization. Good correlation was claimed. Application of this test to any patient showing jaundice or other clinical signs of liver damage after sulfonamides might be of use in establishing

the true mechanism involved, for the evidence at present available is almost entirely circumstantial.

ASSOCIATION WITH LEUKEMIA

In our review of 100 cases of leukemia we found, in addition to the 3 patients with hepatic necrosis, 4 others who had been given one course of sulfonamide, but in whom there was no liver damage. A few other patients in the series may have received sulfonamides, but such a history could not be obtained. Nevertheless, the incidence appears sufficiently high to suggest that the liver in leukemia may be peculiarly vulnerable to damage by sulfonamides. Such susceptibility does not appear related to previous clinically or histologically demonstrable hepatic disease, for Peterson, Deutsch, and Finland[14] (1943) showed that sulfonamides could be given without ill effect to patients with either acute or chronic liver damage. More *et al.*[5] (1946) have suggested, however, that altered metabolism from disease or nutritional deficiency may be a factor in the production of sulfonamide sensitivity. They noted that in the healthy individual given sulfonamide prophylactically complications are negligible, as evidenced by Hodges'[15] (1944) series of 5,000 cases which received the drug as prophylaxis against respiratory disease. Changes in plasma protein have been described in leukemia (Nitshe and Cohen,[16] 1947; Brown, Read, Wiseman, and France,[17] 1948). These changes include diminution in albumin and increase in total globulin with variation in relative amounts of alpha and gamma globulin. Hawking and Laurence[18] (1950) suggested that sensitivity is not to sulfonamides alone, but to a combination of them with plasma or tissue proteins. This is supported by the work of Leftwich[13] (1944), to which we have already referred, and that of Wedum[19] (1942), who was able to sensitize animals to sulfonamide azoproteins made by conjugation with human serum, using the Landsteiner technique. A possible explanation of the association we have found between leukemia and hepatic necrosis following sulfonamide administration is that in leukemia, as a result of altered metabolism, proteins may be modified and conjugate unusually readily with sulfonamide to produce a local sensitivity reaction in the liver.

SUMMARY AND CONCLUSIONS

Three cases of widespread hepatic necrosis following sulfonamide therapy in patients with leukemia are described. The livers from 97 other cases of leukemia were reviewed to exclude leukemia as a direct

cause of the necrosis. The evidence from our cases and those previously reported suggests that hepatic necrosis after sulfonamides is of allergic origin. In leukemic patients there appears to be unusual danger of this complication, particularly if sulfonamides are given in repeated courses, and it seems that penicillin or some other of the antibiotics now available is to be preferred for the treatment of intercurrent infections in such cases.

We should like to thank Dr. L. Stent for permission to use the necropsy material in case 2, Dr. C. S. D. Don, Dr. J. S. Parkinson, Dr. J. F. Wilkinson, and Mr. N. A. J. Young for access to case notes, and Professor A. C. P. Campbell, Dr. M. C. G. Israels, and Dr. F. A. Langley for their helpful criticism and advice.

REFERENCES

1. Cline, E. W. Acute yellow atrophy of the liver following sulfanilamide medication. *J. A. M. A.*, 1938, **111**, 2384–2385.
2. Russell, H. K. Acute toxic necrosis of the liver following the use of sulfanilamide. *Ann. Int. Med.*, 1940, **14**, 168–170.
3. Berger, S. S., and Applebaum, H. S. Toxic hepatitis due to sulfanilamide; report of a fatal case with histopathological findings in the liver. *J. Lab. & Clin. Med.*, 1940–41, **26**, 785–790.
4. Herbut, P. A., and Scaricaciottoli, T. M. Diffuse hepatic necrosis caused by sulfadiazine. *Arch. Path.*, 1945, **40**, 94–98.
5. More, R. H., McMillan, G. C., and Duff, G. L. The pathology of sulfonamide allergy in man. *Am. J. Path.*, 1946, **22**, 703–735.
6. Krusius, F. E. Akute diffuse Lebernekrose nach Sulfathiazolverabreichung. *Ann. med. int. Fenniae*, 1947, **36**, 83–97.
7. Merrill, D. Antibiotics in chronic leukemia. *Blood*, 1948, **3**, 918–920.
8. Lederer, M., and Rosenblatt, P. Death during sulfathiazole therapy; pathologic and clinical observations on four cases with autopsies. *J. A. M. A.*, 1942, **119**, 8–18.
9. Rammelkamp, C. H. Jaundice and the sulfonamide drugs. *Blood*, 1948, **3**, 1411–1415.
10. Erskine, D. Dermatitis from sulphonamide compounds. *Brit. M. J.*, 1939, **2**, 104–107.
11. Rich, A. R. The role of hypersensitivity in periarteritis nodosa as indicated by seven cases developing during serum sickness and sulfonamide therapy. *Bull. Johns Hopkins Hosp.*, 1942, **71**, 123–140.
12. Rake, G., van Dyke, H. B., and Corwin, W. C. Pathologic changes following prolonged administration of sulfathiazole and sulfapyridine. *Am. J. M. Sc.*, 1940, **200**, 353–362.
13. Leftwich, W. B. An intradermal test for the recognition of hypersensitivity to the sulfonamide drugs. *Bull. Johns Hopkins Hosp.*, 1944, **74**, 26–48.
14. Peterson, O. L., Deutsch, E., and Finland, M. Therapy with sulfonamide compounds for patients with damage to the liver. *Arch. Int. Med.*, 1943, **72**, 594–612.

15. Hodges, R. G. The use of sulfadiazine as a prophylactic against respiratory disease. *New England J. Med.*, 1944, **231**, 817–820.

16. Nitshe, G. A., Jr., and Cohen, P. P. Serum protein changes in myelogenous and lymphocytic leukemias and Hodgkin's disease. *Blood*, 1947, **2**, 363–370.

17. Brown, R. K., Read, J. T., Wiseman, B. K., and France, W. G. The electrophoretic analysis of serum proteins of the blood dyscrasias. *J. Lab. & Clin. Med.*, 1948, **33**, 1523–1533.

18. Hawking, F., and Laurence, J. S. The Sulphonamides. H. K. Lewis & Co., Ltd., London, 1950, 335 pp.

19. Wedum, A. G. Immunological specificity of sulfonamide azoproteins. *J. Infect. Dis.*, 1942, **70**, 173–179.

LEGENDS FOR FIGURES

FIG. 1. Case 1. Low-power view to show widespread necrosis of hepatic cells. Hematoxylin and eosin stain. × 120.

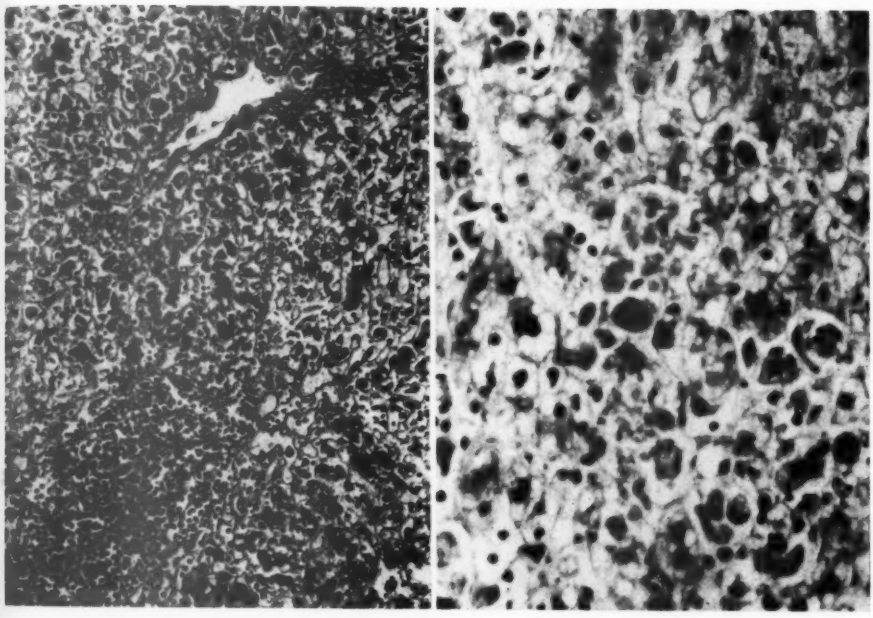
FIG. 2. Case 1. High-power view showing swelling and distortion of surviving liver cells. Hematoxylin and eosin stain. × 320.

FIG. 3. Case 1. Section stained to show large quantities of fat in degenerating liver cells. Sudan IV stain. × 120.

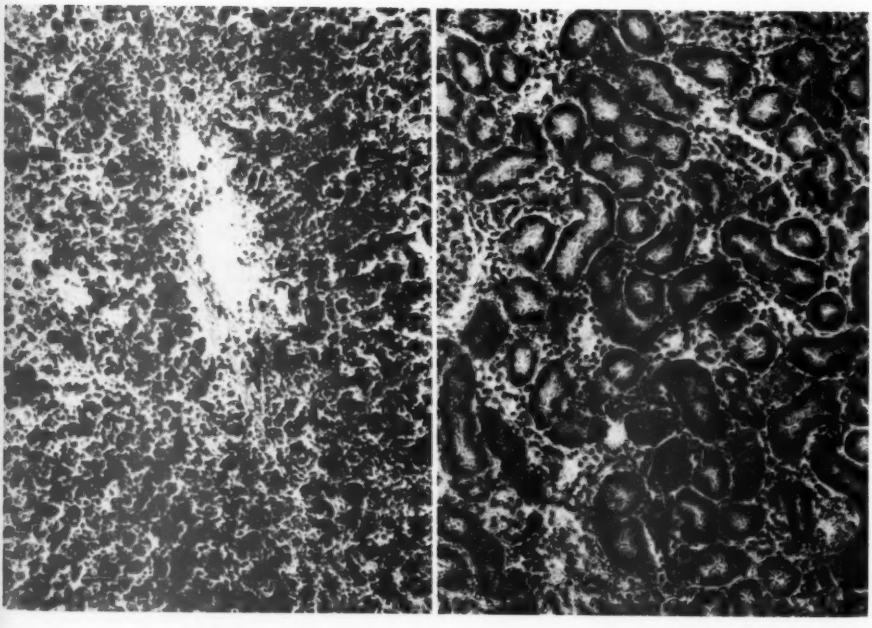
FIG. 4. Case 1. Section stained to show fat in the epithelium of the proximal convoluted tubules of the kidney. Sudan IV stain. × 120.

VOLTAIRE

X



1 2



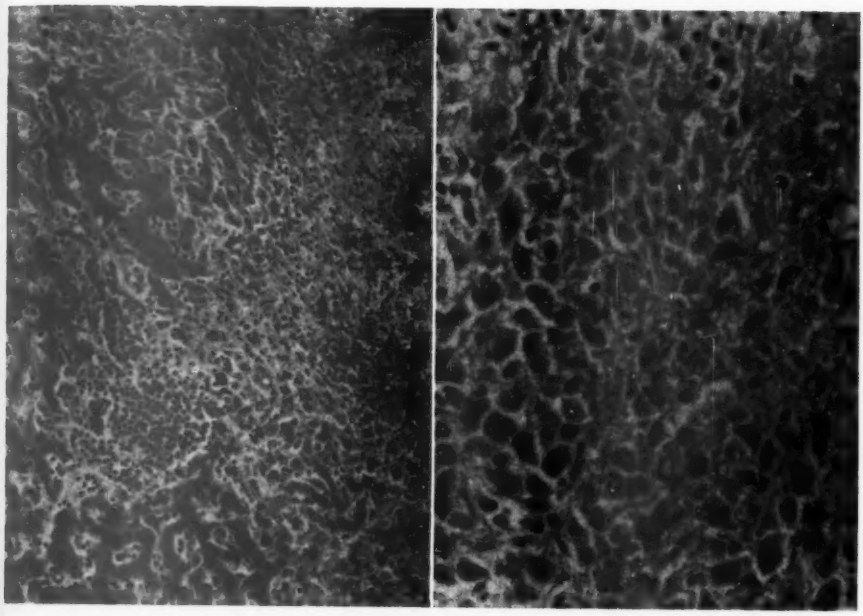
3 4

FIG. 5. Case 2. Focus of necrosis in the liver with hemorrhage and inflammatory cell infiltration. Hematoxylin and eosin stain. × 120.

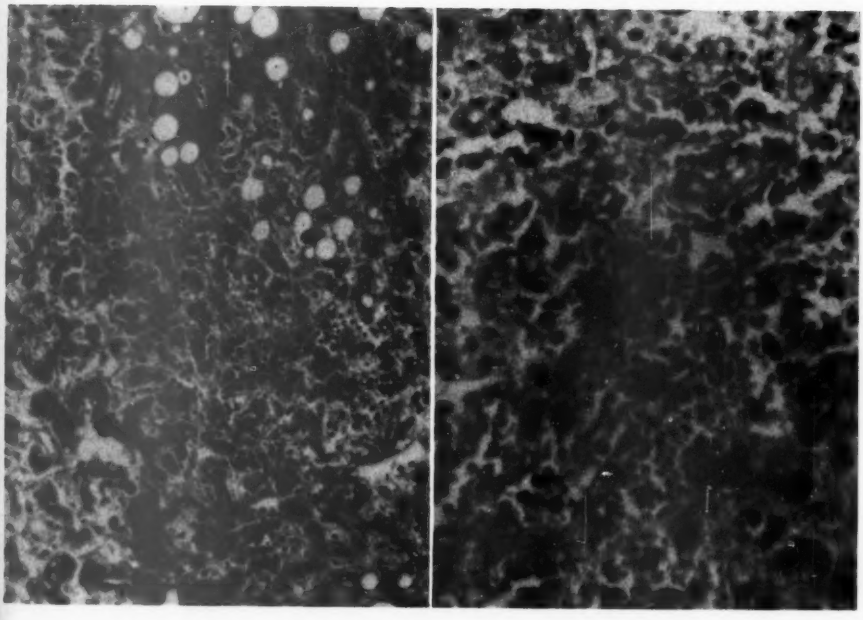
FIG. 6. Case 2. High-power view of the periphery of an area of hepatic necrosis. Hematoxylin and eosin stain. × 320.

FIG. 7. Case 3. Area of hepatic necrosis with polymorphonuclear leukocytic infiltration. Hematoxylin and eosin stain. × 120.

FIG. 8. Case 3. High-power view of a field from the same area as Figure 7. Hematoxylin and eosin stain. × 320.



5 6



7 8

THE INDUCTION OF METASTASES FROM SARCOMA I IN C57BL/6 MICE *

NORMAN MOLOMUT, PH.D., DAVID M. SPAIN, M.D., SIDNEY D. GAULT, B.A., and LEONARD KREISLER, B.A.

(From the Waldemar Medical Research Foundation, 16 Clinton St., Brooklyn 2, N.Y.)

Successful experimental production of what appeared to be intraperitoneal and intrathoracic metastases from a homeograft of Sarcoma I in mice has been observed by us.[1] These metastases resulted after injection of mice with lyophilized neoplastic tissue (intraperitoneally), repeated injections of cortisone, and subcutaneous implantation of living grafts of the same tumor. The present experiments were conducted: (a) to determine whether these tumors in the chest and abdominal cavities were truly metastases, whether they were direct extensions or permeations from the primary graft, or whether the experimental procedure itself permitted the introduction of viable tumor cells by direct injection of lyophilized tumor into the body cavities, and (b) to determine the reproducibility of the phenomenon previously reported.

MATERIALS AND METHODS

Tumor

The tumor used was a transplantable mouse cancer, Sarcoma I, which originated at the University of California in 1947 and on December 3, 1951, was in its 152nd transplant generation at the Jackson Laboratory and has been carried by us since that time in "A" strain mice. This tumor grows in 100 per cent of "A" strain mice and F-1 hybrids, both male and female, when subcutaneously implanted, and is palpable in 2 to 3 days, with the animals dying in approximately 3 weeks with a large tumor mass. The tumor is composed of solid, dense sheets of predominantly polyhedral cells and to a lesser extent of spindle cells with scant stroma. This tumor ordinarily does not grow progressively in untreated C57BL/6 mice.

Mouse Strains[2]

All mice were 8 to 10 weeks of age at the start of treatment and groups were equally divided as to sex (virgin females), housed 5 or 6

* This work was supported by grants from the United States Public Health Service, National Institutes of Health (Grant no. C1658) and the American Cancer Society (recommended by the National Research Council, Committee on Growth) (Grant no. CP54). A portion of this work was performed at the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and the Westchester County Department of Laboratories and Research, Valhalla, N.Y.

Received for publication, July 17, 1953.

per cage, fed on Purina fox chow supplemented with aqueous dispersion of multivitamins, and given water *ad libitum*.

The strain used for the experimental groups was C57BL/6 and the tumor control mice were "A" strain. The tumor was carried in the "A" strain mice as a source of viable graft in all groups.

Preparation of Lyophilized Tissues

Sarcoma I was harvested under aseptic conditions from "A" strain mice (male and female) on the 10th to 14th day after implantation by trochar subcutaneously in the suprascapular region. Only healthy solid neoplastic tissue was used. The harvested tissue was partially minced with scissors, and was transferred to sterile capsule vials with screw caps, which had been previously cooled by imbedding in a dry ice-acetone bath. The partially filled vials were immediately replaced in the freezing bath. The approximate time from harvest to freezing each vial was 5 minutes. When all vials were collected, the entire batch was placed in a freeze-drying apparatus (lyophilizer) and the pressure reduced to below 1 mm. Hg within 15 minutes. The tissues were dried overnight (12 to 16 hours) and the vials removed from the apparatus, capped, tested for sterility, and stored in the deep freeze (or dry ice chest) at approximately −15° to −20° C. from 10 days to several months, as needed. In each of the experiments the control and test groups of animals received lyophilized tissue from the same batch.

For use in the following experiments, the dried material was ground in a sterile glass mortar and then homogenized in distilled water in a small Waring blendor or a ground glass tissue grinder so that 15 mg. of dry weight were contained in 0.5 ml. The resuspended lyophilized tissues were kept in a salt-ice bath during the injection.

In those groups receiving cortisone, the dose was given subcutaneously once daily in a volume of 0.1 ml. away from the tumor graft site and the dose was varied between 0.1 to 1.0 mg. per mouse daily in order to maintain a debilitated state. Bacteremia was controlled by prophylactic intraperitoneal injections of three mixed antibiotics (300 units of penicillin, 0.2 mg. of streptomycin, 0.2 mg. of terramycin) given 10 days prior to the start of the experiment and 1 week prior to live graft implantation.

The tumor grafts were followed by palpation, and at death or sacrifice were examined grossly. When necessary, histologic sections were made (formalin fixation, hematoxylin and eosin stain).

All animals at death or sacrifice were subjected to complete necropsy

and preserved intact in formalin (10 per cent) and histologic sections were made to determine the presence or absence of tumor metastases in the various organs and body cavities. The data to be given are based entirely upon microscopic determination of the tumor as present or absent. The distinction between granuloma and tumor was based on histologic examination.

Experimental Groups and Results

Group 1. Lyophilized Sarcoma I Plus Cortisone Plus a Live Graft

A total of 134 C57BL/6 mice were treated (in 4 successive experimental trials to check the reproducibility of the findings) with cortisone continuously throughout the experiment, varying the dose as indicated by the condition of the animals from 0.1 to 1.0 mg. daily. Cortisone treatment was begun 3 days prior to the first injection of lyophilized Sarcoma I tissue.

An aliquot of each batch of lyophilized Sarcoma I, when resuspended for injection as has been described, was prepared for histologic examination to check for microscopically visible intact cells. None was found (Fig. 1) but occasional pyknotic remnants, singly or in clusters, were seen. The resuspended, homogenized, lyophilized Sarcoma I was given intraperitoneally in three injections, each of 15 mg. in 0.5 ml. volume, at 5 day intervals for a total 45 mg. dry weight of tissue per mouse. Ten days after the last injection of lyophilized Sarcoma I a live graft of a Sarcoma I fragment from a freshly sacrificed "A" strain, tumor-bearing mouse was grafted by trochar subcutaneously in the suprascapular region. Care was taken to assure subcutaneous implantation of a single fragment as close to the head as possible. Forty-one mice of the total of 134 died during the course of experiment prior to live graft implantation and are therefore not included in these data. Ninety-three animals survived the critical portions of the procedure and were sacrificed on successive days starting on the 14th day after implantation of the graft when several mice were noted with markedly distended abdomens. From that time on, mice died and/or were sacrificed every other day until the 24th day after the live graft.

Twenty-eight of the 93 mice had, to a varying extent, intraperitoneal sarcomatosis (Fig. 2) involving mesentery, serosa of intestine (Fig. 3), mesenteric and inguinal lymph nodes, pancreas (Fig. 4), diaphragm, and perirenal space. In 3 additional mice, sarcoma was found in the visceral pleura and in the parenchyma of the lungs (Fig. 5).

In the 31 mice with metastases, the primary graft grew to a size of approximately 1.0 cm. in longest diameter and remained static in 2 until sacrifice; in 7 mice, the graft grew progressively (Fig. 6) and varied with time of sacrifice from 2.5 to 3.5 cm. in diameter; in 22 mice, the primary graft regressed after reaching a maximum of 1.0 to 1.5 cm. (Fig. 7) and was small and necrotic at death or sacrifice (Table I).

Many of the 93 mice, both with and without metastases, contained granulomatous nodules in the peritoneal cavity, which were sterile on bacteriologic culture, and on histologic examination revealed necrotic and amorphous tissue surrounded by a fibrous capsule infiltrated with lymphocytes, plasma cells, and macrophages.

Metastatic sarcoma appeared in approximately as many females as

TABLE I

Relation of Metastases to Growth of Primary Implant

State of graft at time of sacrifice or death	Lyophilized sarcoma plus cortisone plus graft		Lyophilized sarcoma plus graft no. of mice	Cortisone plus graft no. of mice
	No metastasis no. of mice	With metastasis no. of mice		
Growing progressively	34	7	10	0
Static	17	2	11	0
Regressing	11	22	6	30
Total number of mice	62	31	27	30

males and was identical in histologic appearance with the primary graft. Careful gross and histologic examination of the body tissues between the primary graft and the body cavities showed no evidence of direct extension of tumor in the 31 animals described. Several mice with metastases were not included in the group of 31 because microscopic evidence of sarcoma in the tissue layers below the primary graft made an opinion of direct extension possible.

Most of the 31 mice with metastases in the abdomen had ascites.

The viability and growth characteristics of the metastatic sarcomata were checked by transplantation to normal mice of varying strains and have been reported.[3]

Control Group II

Fifty C57BL/6 mice were treated according to the foregoing procedures with cortisone and lyophilized sarcoma, but no live graft was implanted. Nine of this group died prior to the third injection of lyophilized Sarcoma I. The remainder of the animals were sacrificed in parallel with the experimental group I, so that mice were examined at the same time interval after the last dried tissue injection. None

of these mice showed either gross or microscopic evidence of tumor in any of the body cavities. Granulomas were present.

Control Group III

Thirty C57BL/6 mice received only lyophilized Sarcoma I and a live Sarcoma I graft as in group I. Three died prior to the graft and so were discarded. The remaining 27 mice were sacrificed to parallel group I. At the time of sacrifice the tumor was growing progressively in 10 mice, having reached 2.5 cm. in size on the 21st day; in 11 mice the tumor was 1.0 cm. by the 8th to 10th day and remained so until sacrifice; in 6 mice the tumor reached a maximum of 1.0 cm. by the 8th to 10th day and was regressing at the time of sacrifice. None of these animals showed evidence of metastases when examined as previously described. (See Table I.)

Control Group IV

Thirty C57BL/6 mice were treated with cortisone only and implanted with Sarcoma I graft as in group I. The tumor reached an average maximum size of 1.0 cm. in all 30 of these mice by the 7th to 10th day, at which time the primary grafts were necrotic and no evidences of metastases were found. (See Table I.)

Control Groups V and VI

Tumor viability controls: Twenty C57BL/6 and 20 "A" strain mice of the same age and sex distribution as for the preceding groups were grafted with Sarcoma I with no prior treatments. The Sarcoma I graft grew progressively and all 20 of the "A" strain mice died with large tumors. The graft regressed in all 20 of the C57BL/6 mice. No evidence of metastases was found in any of the mice in either of these groups.

Discussion

The variation in time of development of metastases after the appearance of a primary malignant tumor of the same histogenesis and cytology in humans (*i.e.*, in melanoma from 6 months to 15 years or more) led us to investigate the possible factors involved in the host's resistance to the spread of malignant tumors. Casey[4,5] and Kaliss and Snell[6,7] have shown that prior injections of mice or rabbits with lyophilized cancer or normal tissue from the strain of mice in which the intended cancer graft was indigenous enhanced the growth of a graft in a strain so treated, even if ordinarily it would not grow the graft. These investigators believed that there is some substance or group of substances in the injected material which acts as an enhancing

factor. However, Kaliss and Molomut[8] have demonstrated that this phenomenon occurs as well with injections of the anti-serums to the lyophilized tissues prepared in either rabbits or mice. In the current studies it has been demonstrated that the injected lyophilized tissue produces marked granulomatous responses at the sites of injection and therefore acts as a local or systemic trauma to the host. For this reason and because the anti-serums also affect tumor growth, it is our belief that the effect is primarily a non-specific one on the host's resistance to the tumor rather than depending on any specific group of enhancing substances in the injected material. The degree of specificity or non-specificity of the enhancing effect still requires investigation before final decision is possible.

In previous studies it has been demonstrated that cortisone interferes with the host's local and systemic responses to the introduction of foreign agents, whether these be viruses, bacteria, chemical agents, or foreign living bodies, such as tumor grafts.[9-11] This provided us with a convenient test by which we could study the effect of the trauma induced by lyophilized tissue injections combined with inhibition of the host's responses by cortisone on the growth of tumor transplants. This combination in the group I mice resulted in the development of intraperitoneal sarcoma (metastases) and, on occasion, intrathoracic sarcoma (metastases) in 31 (33 per cent) of 93 animals. This result was reproducible in four different trials with separately prepared batches of lyophilized sarcoma.

Currently, investigation is being conducted with a different tumor in a different strain of mice to determine whether this phenomenon is general or applicable only to the set of circumstances peculiar to the reported experimental procedure.

The failure of intraperitoneal sarcoma (metastases) to develop in those animals that received only lyophilized sarcoma and cortisone and in which no live graft was implanted is evidence against the possibility that the intraperitoneal tumors arose from refractory but viable cells in the lyophilized Sarcoma I. This possibility must be considered seriously because of Craigie, Hayward, and Begg's[12] reports on the development of viable tumor in mice from the injection of lyophilized neoplastic tissue. It is important to note that there is a considerable difference in the technique of lyophilization as performed by Craigie and by us. Ours is a much cruder technique, with the drying procedure extending for 14 hours, and would be less likely to preserve the viability of the cells. Histologic examination of the lyophilized sarcoma failed to reveal cells which we could consider to be intact. The presence of tumor in the intrathoracic cavity, despite the facts that lyo-

philized tissue was not injected into this region and that the tumor did not develop in the peritoneal cavity, is also against the origin of the tumor from the lyophilized tissue. However, further varied and controlled experiments are necessary to establish this point.

The next question that arose was whether the neoplastic masses were true metastases or merely the result of direct extension from the primary graft. The fact that in most instances intraperitoneal metastases were present in animals in which the primary graft was small or regressing is in favor of true metastases. In contrast, those animals in the various groups which had tremendous tumors growing downward with actual invasion of the abdominal wall usually showed no evidence of intraperitoneal or intrathoracic malignant tumor. Histologic sections of the tissues between the primary graft and intraperitoneal metastases confirmed the fact that no direct extension was present. An additional finding in support of this view were the isolated nodules of Sarcoma I within the pulmonary parenchyma in the absence of invasion of the pleural space. The possibility that some virus-like factor present in the Sarcoma I was enhanced by the use of cortisone must be considered. To our knowledge, no such virus factor has been demonstrated as associated with Sarcoma I. However, this requires further investigation.

A recent report[13] of induced metastases of homologous cancer graft in C_3H mice by cortisone injections supports our data. These investigators attributed their findings to a tumorigenic action of the cortisone despite their finding that cortisone delayed and arrested the primary cancer implant. It is also significant that the C_3H mouse mammary tumor has been associated definitely with a virus-milk factor and it is known that cortisone increases susceptibility to viral and bacterial agents. In the current investigations it was noted that metastases occurred most frequently in those instances in which the primary tumor graft was regressing. This certainly is against the concept that cortisone acts as a tumorigenic agent. The alterations in the tumor bed itself, the effect of these procedures on the homologous strain, and the effect of ACTH in place of cortisone are all under investigation at the present time.

Summary

An inbred strain of mice ($C_{57}BL/6$) maintained on cortisone and treated with intraperitoneal injections of lyophilized neoplastic tissue (Sarcoma I) developed intra-abdominal and intrathoracic metastases after implantation with a live graft of the Sarcoma I, which ordinarily does not grow in the $C_{57}BL/6$ mouse strain.

In the treated mice in which metastases occurred (33 per cent of

the total) the primary live tumor graft regressed, while in those mice (treated and control) without metastases the primary graft grew progressively in a significantly larger number.

Photomicrographs by Robert Duckworth of the Westchester Cancer Committee. Cortisone was obtained from Merck & Co., Inc., Rahway, N.J., in the form of a sterile suspension of "Cortone Acetate." Inbred lines of mice were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

REFERENCES

1. Molomut, N., Spain, D. M., Gault, S. D., and Kreisler, L. Preliminary report on the experimental induction of metastases from a heterologous cancer graft in mice. *Proc. Nat. Acad. Sc.*, 1952, **38**, 991–995.
2. Standardized nomenclature for inbred strains of mice, prepared by the Committee on Standardized Nomenclature for Inbred Strains of Mice. *Cancer Research*, 1952, **12**, 602–613.
3. Spain, D. M., Molomut, N., Kreisler, L., and Gault, S. D. Histopathology of host-induced alterations in the strain specificity of sarcoma I in mice. *Am. J. Path.*, 1953, **29**, 933–941.
4. Casey, A. E. Experimental enhancement of malignancy in the Brown-Pearce rabbit tumor. *Proc. Soc. Exper. Biol. & Med.*, 1931–32, **29**, 816–818.
5. Casey, A. E. Specificity of enhancing materials from mammalian tumors. *Proc. Soc. Exper. Biol. & Med.*, 1933–34, **31**, 663–665.
6. Kaliss, N., and Snell, G. D. The effects of injections of lyophilized normal and neoplastic mouse tissues on the growth of tumor homiotransplants in mice. *Cancer Research*, 1951, **11**, 122–126.
7. Kaliss, N. Effect of prior injection of non-mouse tissues on growth of tumor homoiografts in mice. *Science*, 1952, **116**, 279–280.
8. Kaliss, N., and Molomut, N. The effect of prior injections of tissue antiserums on the survival of cancer homoiografts in mice. *Cancer Research*, 1952, **12**, 110–112.
9. Spain, D. M., and Molomut, N. Effects of cortisone on the development of tuberculous lesions in guinea pigs and on their modification by streptomycin therapy. *Am. Rev. Tuberc.*, 1950, **62**, 337–344.
10. Shwartzman, G. Enhancing effect of cortisone upon poliomyelitis infection (strain MEF1) in hamsters and mice. *Proc. Soc. Exper. Biol. & Med.*, 1950, **75**, 835–838.
11. Spain, D. M., Molomut, N., and Haber, A. Studies of the cortisone effects on the inflammatory response. I. Alterations of the histopathology of chemically induced inflammation. *J. Lab. & Clin. Med.*, 1952, **39**, 383–389.
12. Craigie, J., Hayward, M. E., and Begg, A. M. Preliminary observations on a "dormant" state of sarcoma cells with special reference to resistance to freezing and drying. *J. Path. & Bact.*, 1951, **63**, 177–178.
13. Agosin, M., Christen, R., Badinez, O., Gasic, G., Neghme, A., Pizarro, O., and Jarpa, A. Cortisone-induced metastases of adenocarcinoma in mice. *Proc. Soc. Exper. Biol. & Med.*, 1952, **80**, 128–131.

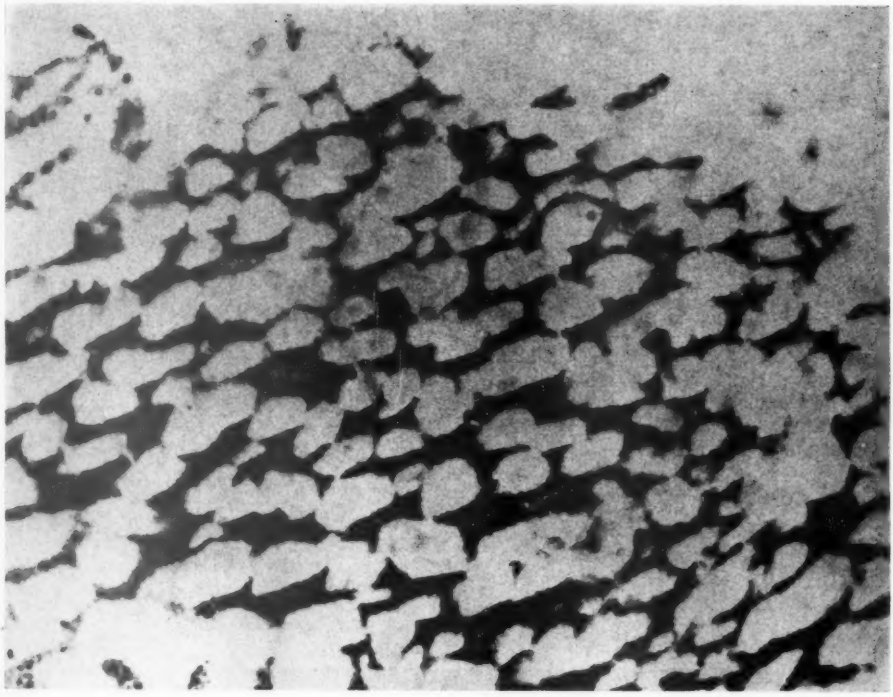
[*Illustrations follow*]

LEGENDS FOR FIGURES

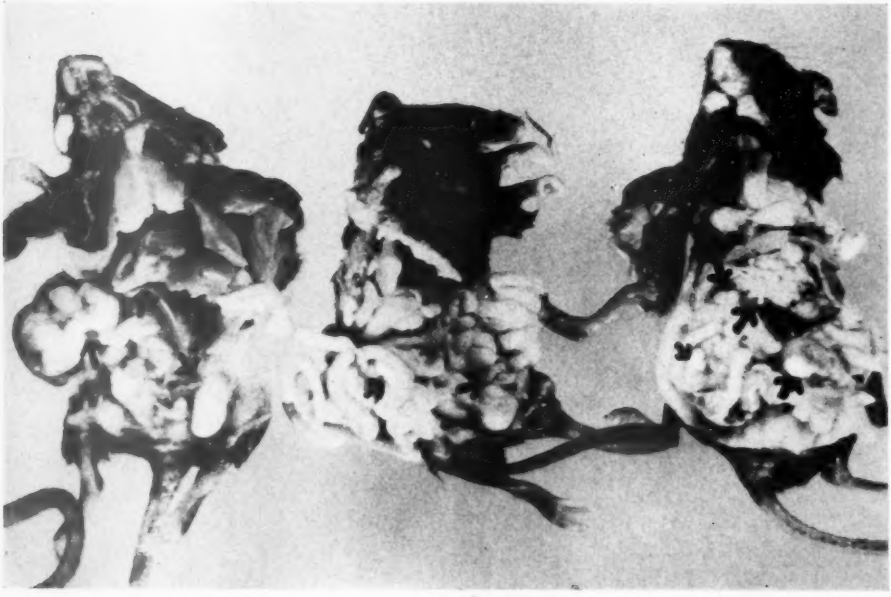
FIG. 1. Photomicrograph showing the appearance of lyophilized Sarcoma I. A few pyknotic cells are present. Hematoxylin and eosin stain. × 250.

FIG. 2. Photographs of 4 C57BL/6 mice treated with lyophilized Sarcoma I and cortisone, and implanted with a live Sarcoma I graft. Arrows point to tumor masses which were microscopically identical to Sarcoma I that was implanted in the supra-scapular region.

1



2

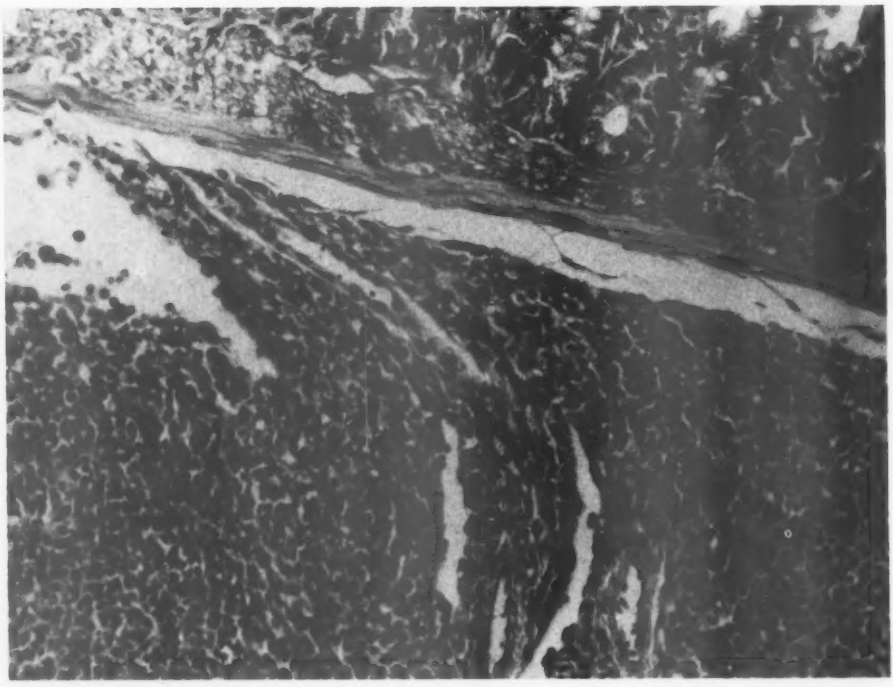


a b c

FIG. 3. Photomicrograph of a section from the nodule involving the small intestine in the mouse shown in Figure 2 (b). Hematoxylin and eosin stain. × 250.

FIG. 4. Photomicrograph of a section showing infiltration of Sarcoma I in the pancreas in the mouse shown in Figure 2 (c). Hematoxylin and eosin stain. × 250.

FIG. 5. Photomicrograph showing Sarcoma I surrounding a bronchus in a C57BL/6 mouse. There is uninvolved lung between the tumor and the pleural surface. Hematoxylin and eosin stain. × 250.



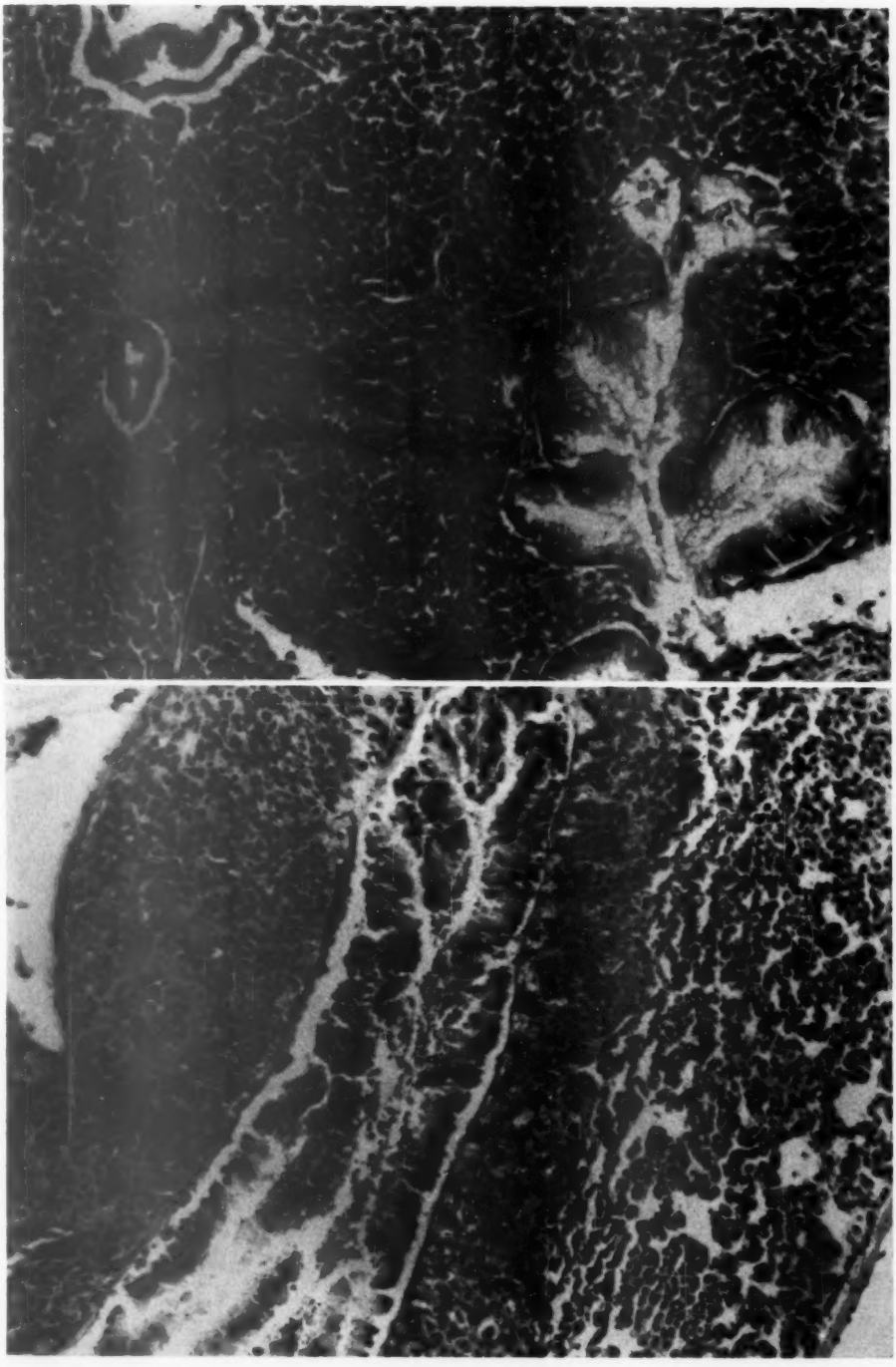
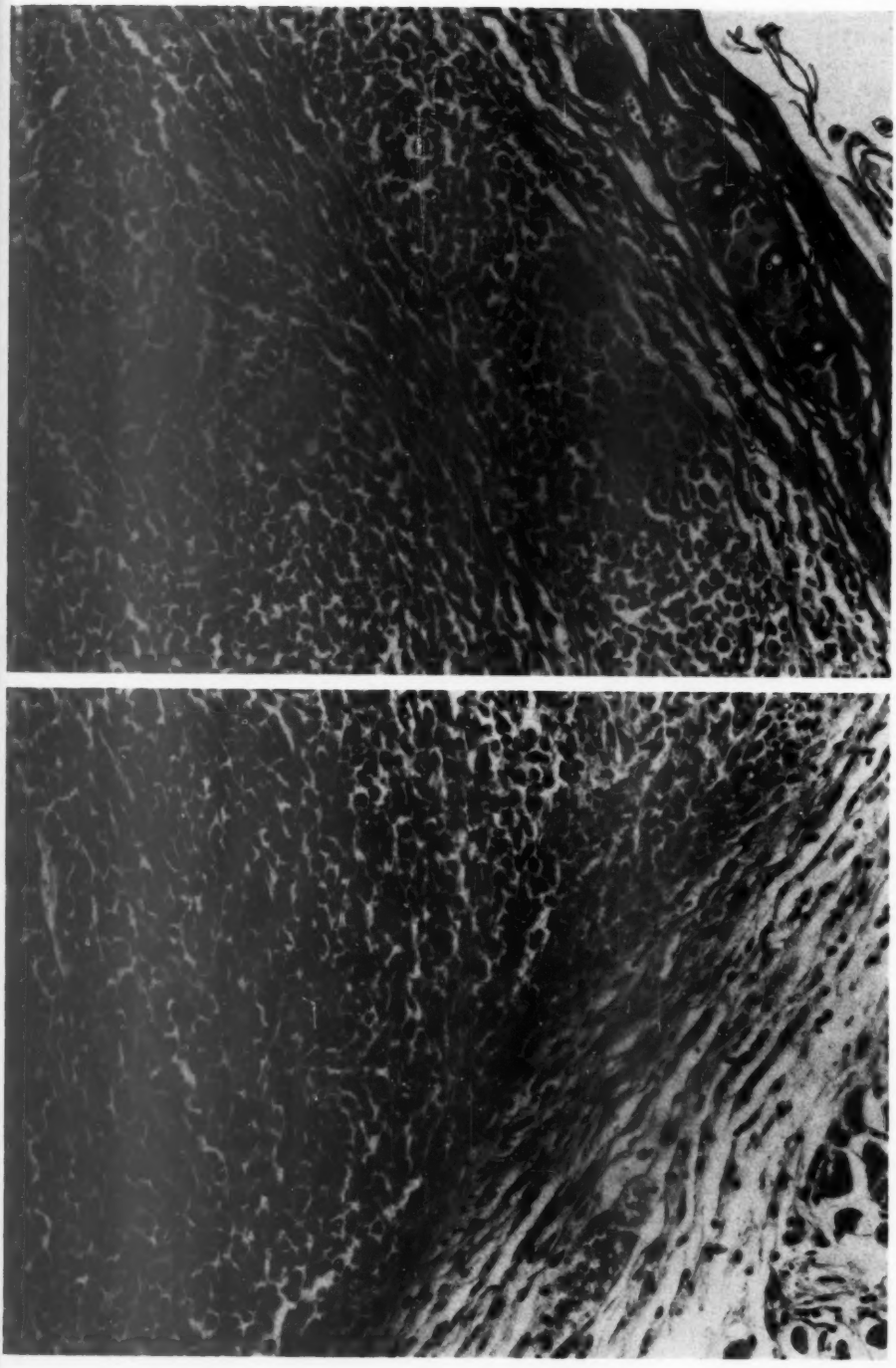


FIG. 6. Photomicrograph showing Sarcoma I at the implant site in a C57BL/6 mouse treated with lyophilized Sarcoma I, cortisone, and a live graft. This tumor grew progressively and was 3.0 cm. in diameter at the time of sacrifice. Hematoxylin and eosin stain. × 250.

FIG. 7. Photomicrograph of mouse treated as in Figure 6, but which was regressing at the time of sacrifice. Hematoxylin and eosin stain. × 250.



MORPHOLOGY OF TISSUE MAST CELLS

The Frequency of Artifacts and the Influence of Certain Biologic Agents *

James E. Devitt, M.D., Peter B. Samuels, M.D.†, W. J. Pirozynski, M.D., and D. R. Webster, M.D.

(From the Department of Experimental Surgery, McGill University, Montreal, Que.)

Since Ehrlich[1] described the mast cells in 1879, much confusing and controversial material has been reported on the nature and function of these ubiquitous cells. Currently they are thought to have a secretory function, but whether the secretion is heparin,[2] hyaluronic acid,[3] or histamine[4] is disputed. These theories have been based largely on morphologic appearances which have been interpreted as representing functional or degenerative changes concomitant with secretory activity. Probably the earliest microscopic finding that led to speculation as to a possible secretory function was the appearance of extracellular nongranular metachromatic halos and hazes. Harris[5] and Michels[6] demonstrated thoroughly, however, that these halos and hazes were artifacts due to the use of water in the preparation of the tissue, which dissolved the readily soluble granular substance. Another suggestive finding has been the frequent occurrence of metachromatic granules scattered outside the cell bodies. Michels pointed out that this was due to rupture of the cell body, either by the microtome knife or by too rapid fixation when sudden uneven shrinkage of the cell occurs. However, old legends die hard, and many contemporary authors still seem to regard cell rupture with granule scattering as the mechanism by which the granule content of mast cells is delivered to the surrounding tissue.

Wilander,[2] studying dogs in peptone shock, observed in the liver mast cells in which only the peripheral granules were stained. He interpreted this as indicating that these mast cells were secreting their alleged heparin content.

Paff and Bloom[7] let cultures of mast cell tumors age, and observed what they described as degenerative changes (vacuolation, granule dissolution, and discharge), and very occasionally, metachromatic substance outside a few cells. This they thought represented heparin discharge. They believed that the secretory cycle of heparin production involved degenerative changes and even death of the mast cells. Similar

* Supported by a grant from the Cancer Research Society, Inc., Montreal, Que.
Received for publication, August 22, 1953.
† United States Public Health Service Fellow.

morphologic findings were observed by Camble[8] following the injection of toluidine blue into rats.

Drennan,[9] by inducing edema in the lesions of urticaria pigmentosa, claimed that the mast granules became irregular and clumped into large sheets with the appearance of cytoplasmic patches free from metachromasia. Ultimately the cells burst. He believed that this indicated the mechanism of heparin release.

Asboe-Hansen[3] has correlated the absence of hyaluronic acid in the subcutaneous connective tissue during thyrotoxicosis with the small granule-poor mast cells present. Cavallero and Braccini,[10] Asboe-Hansen,[11] Bloom,[12] and Stuart[13] have reported that cortisone induces cytoplasmic vacuolation, granule conglomeration, and altered staining reaction of the metachromatic granules, disappearance of many fine granules, fraying and disruption of the cellular membrane, granule scattering, and finally cellular destruction and disappearance. Baker, Ingle, and Li,[14] on the other hand, failed to observe such changes. Although Cavallero and Braccini[10] and Asboe-Hansen[11] correlated these changes with the diminution of hyaluronic acid in the connective tissue following cortisone administration, these phenomena have been brought forward also by those considering the mast cells as the site of heparin production.

Smith and Lewis[15] observed conglomeration of cytoplasmic granules, vacuolation, polymorphism, and rupturing of mast cells following total body x-irradiation. They interpreted these changes as representing various stages of mast cell degeneration with eventual heparin liberation.

Riley and West[4] observed bursting of the mast cells following administration of a histamine liberator and regarded this alteration as representing histamine release. Baeckeland[16] has described degenerative changes in mast cells engaged in the lysis of an implanted blood clot. Other authors[13,17] described variations in number of granules, in the degree of granular metachromasia, and in the amount of material present in the cell.

. Cramer and Simpson[18] described mast cells with diminished numbers of granules and orthochromatic substance resulting from the use of a watery fixative. In addition Compton[19] found granule dissolution and vacuolation to be rare in fresh connective tissue spreads. These observations, however, seem to have been largely ignored.

The foregoing statements make it apparent that the present theories of mast cell function are based to some extent on morphologic appear-

ance of the cells. Until the reliability of these criteria is established, it is hazardous to base theoretic considerations upon them.

This study, therefore, was undertaken to determine the morphologic variations in mast cells under physiologic conditions and stimuli. Furthermore, it was believed desirable to compare these physiologic changes with those following treatment with biologic agents. Finally, an attempt was made to assess the importance of artifact in the production of the morphologic picture.

Materials and Methods

Male piebald rats averaging 225 gm. in weight were used throughout. The morphologic presentation of tissue mast cells was studied in skeletal muscle, heart muscle, and mesentery of approximately 60 control animals. Specimens of heart and skeletal muscle were fixed in Carnoy's fluid, cut in serial sections 7 μ thick, and stained with 0.5 per cent toluidine blue in a 30 per cent alcoholic solution. The mesentery was studied using a whole mount technique.[20] After fixation and staining, the specimens were dehydrated in a mixture of equal parts of ethanol and of butanol for 1 hour and in pure butanol for ½ hour. They were cleared in a mixture of 4 parts xylol and 1 part methyl salicylate for about 1 to 15 minutes, after which the mesentery was trimmed from the loop of ileum and mounted on the slide in the usual manner. All specimens were removed from rats anesthetized by ether inhalation before death ensued.

In another group of 90 animals the influence of various biologic agents on the structure and number of mast cells was investigated. The following substances were administered to various groups of rats:

1. Thyroxine (Squibb)* was injected subcutaneously in a dose of 200 μg. per day for 7 days and in another group as a single dose of 600 μg.

2. Desoxycorticosterone acetate (Ciba)* in sesame oil was given in a daily dose of 10 mg. for 3, 6, and 8 days.

3. Cortone acetate (Merck)* was injected subcutaneously in a daily dose of 5 and 10 mg. for 6 days.

4. Heparin (Organon)* was given subcutaneously in a dose of 100 mg. twice daily for 6 days.

5. A single dose of 2.5 mg. of "48-80" (a condensation of *p*-

* E. R. Squibb & Sons, New York, N.Y., Ciba Pharmaceutical Products, Inc., Summit, N.J., Merck & Co., Inc., Rahway, N.J., and Organon, Inc., Orange, N.J.

methoxy-phenyl-ethyl-methylamine with formaldehyde), a potent histamine liberator, was given to another group of rats.

6. Hyaluronidase (Schering)* was given in the dose of 150 turbidity reducing units twice daily for 2 days intraperitoneally with the fifth dose 1½ hr. before sacrificing.

All animals were sacrificed 24 hours after the last injection. Specimens of heart, skeletal muscle, and mesentery were investigated using the same technique as for controls.

In 60 controls and 90 treated animals approximately 250,000 tissue mast cells were examined.

To avoid the influence of the fixation-staining-mounting technique, a group of 20 rats was studied by examination of mast cells *in vivo*. The rats were anesthetized by subcutaneous injection of sodium pentobarbital; loops of small bowel were pulled out and kept moist with normal saline solution at 38°C. Staining of the mesentery was accomplished by adding 0.025 per cent toluidine blue solution in saline. The preparation was then examined under the water immersion lens.

Observations and Results

After careful scrutiny of the fixed specimens from control animals it became apparent that all alterations previously associated with physiologic change or fixation artifact were equally as numerous as "normal" cells; indeed, the mast cell with discrete metachromatic granules filling the cell was relatively uncommon.

The following types of "degeneration" could be seen in the majority of tissue mast cells in a specimen by varying the technique used:

Staining Reaction. Over-staining with toluidine blue resulted in blue-black coloration of chromotropic material with little if any evidence of metachromasia. Adequate staining, under-staining, or washing out of the stain with alcohol resulted in metachromatic staining of the mast cells. If under-staining or washing out was carried to an extreme, the mast cell might become faded and almost impossible to identify.

Amount of Chromotropic Substance. Generally, over-staining gave the mast cells the appearance of overloading with chromotropic substance (Fig. 1), while under-staining or washing out with alcohol caused the cells to appear poorly filled (Fig. 3).

Granule Conglomeration. Granule conglomeration was most apparent in moderately over-stained specimens (Figs. 4 and 8). In overstained specimens the mast cells appeared almost completely solid with a scattering of granules (Fig. 8). When specimens were understained the granules tended to be discrete and gave the impression of

* Schering Corporation, Bloomfield, N.J.

even distribution (Fig. 2). Although agglutinated granules were observed in these specimens, definite clumping with bar formation, as seen in Figure 5, was not observed unless there was some degree of over-staining.

Cytoplasmic Vacuolation and Fraying of Outlines. The phenomenon of cytoplasmic vacuolation and fraying of outlines was most clearly seen in over-stained specimens (Fig. 5). Often a central mass of chromotropic substance enclosing the nucleus was surrounded by a clear area which in turn was encircled by granules.

Granule Scattering. Granule scattering was seen commonly, especially in over-stained specimens (Fig. 5). The burst cells almost invariably contained large, irregular groups of granules, some of which were agglutinated. In under-stained or washed out preparations the burst cells contained distinct and discrete granules (Fig. 6). The granules of the burst cells might lie in a compact group only slightly larger than the intact cell. Frequently, however, the granules were widely separated and unevenly distributed over an area three or four times the original cell area.

Metachromatic Halos. Metachromatic halos were not observed in this study, though they were frequently seen in another study of dog mast cells carried out in this laboratory. It is probable that the absence of halos is accounted for by a solubility difference in the granules of the rat mast cell, as pointed out by Michels.[6]

The administration of such agents as thyroxine, cortisone, desoxycorticosterone acetate, heparin, histamine liberator, or hyaluronidase did not result in the production of changes unlike those observed in the control animals. Morphologic alterations were observed with the same frequency as in in the controls. These changes were: staining aberrations, granule conglomeration, vacuolation, and granule scattering. Neither increase nor decrease in the number of mast cells was seen in any of the groups of treated animals. Statistical support of these observations has been presented elsewhere.[20]

The mast cells observed ***in vivo*** contrasted strongly with those in the fixed control specimens. At no time were appearances suggesting artifact or degeneration seen in the living mast cell. However, as soon as local circulatory stasis or death of the animal occurred, irregularities in the staining and distribution of granules were noted. These changes were seen to result in the eventual disintegration of the cell.

Disturbances of osmotic and chemical equilibria were produced by application of distilled water or methanol on the surface of the mesen-

tery. Almost immediately following the application of these substances the mast cells became swollen, granules were deranged, and the cell subsequently burst (Fig. 10).

Under the more optimal conditions represented by the *in vivo* preparation, the changes in mast cell morphology usually seen in the fixed state were never observed (Fig. 9).

In the review of the literature a number of theories of mast cell function were presented briefly. These theories were based on morphologic changes in the mast cells. In the present study these changes have been shown to occur in the fixed specimens with equal frequency in both controls and animals treated with biologic agents. On the other hand, these changes have not been observed in the *in vivo* preparations studied.

In addition, enumeration of the mast cells in control and treated animals involves no significant differences in number. The morphologic appearance of the mast cell following current techniques of fixation and staining apparently does not reflect the functional state of the cell. This is in disagreement with observations reported by Asboe-Hansen,[3,11] Bloom,[12] Cavallero and Braccini,[10] and Stuart,[13] in which morphologic and numerical changes were described following the administration of cortisone, thyroxine, and desoxycorticosterone acetate.

Cramer and Simpson[18] have suggested that these artifacts are indications of differing functional states in the cells just prior to fixation. In support of this thesis is the frequent finding of closely adjacent cells whose morphology is completely disparate. The fixation artifacts, however, cannot be correlated with any known functional state in the mast cell and thus their use as a definite criterion is unjustified.

Wilander[2] demonstrated that there is a correlation between heparin content of a tissue and the number of mast cells therein. He believed that the mast cell secretion of heparin took place in the form of granule dispersion. Since this study has demonstrated that granule scattering occurs with equal frequency in control and treated animals, and since it can be produced *in vivo* by appropriate treatment, it is considered that mast cell dispersion is not necessarily correlated with heparin secretion.

Recently Schoch and Glick[21] reported a study of mast cells in the skin of the rat. These workers used a counting technique on full thickness biopsy specimens of skin, and also a subcutaneous spread method. They observed the effect of cold stress, ACTH, and cortisone on the mast cell numbers and morphology. They were unable to establish any consistent changes in the concentration or structure of skin mast cells in the treated animals as compared with those in controls. The results

of these investigations on skin mast cells seem to correlate exactly with the findings for other organs and tissues of the rats as reported in this study.

Summary and Conclusions

Morphologic features of tissue mast cells were studied in the skeletal muscle, heart, and mesentery of the rat. A whole mount technique was employed for the mesentery, while the skeletal muscle and heart were prepared by the usual histologic procedures. All were stained with a solution of toluidine blue in aqueous alcohol. Thyroxine, desoxycorticosterone acetate, cortisone, heparin, hyaluronidase, and a histamine liberator were administered to groups of rats. So-called degenerative changes were observed routinely in all specimens, and were found to be no more common in treated animals than in controls. The morphologic appearances can be closely correlated with technical influence. Examination *in vivo* of carefully prepared mesenteries fails to reveal so-called degenerative changes unless unnatural factors are introduced.

It seems, therefore, that in ordinary histologic preparations the morphologic features of the tissue mast cell are too variable and artifacts too frequent to allow any valid interpretations.

We wish to express our thanks for generous donations of desoxycorticosterone acetate from Ciba Pharmaceutical Products; of cortisone from Dr. K. R. MacKenzie of the University Clinic of the Royal Victoria Hospital, Montreal; of heparin from Dr. Kenneth Thompson, Medical Director of Organon, Inc.; and of "48-80" from the Department of Physiology, McGill University, Montreal.

REFERENCES

1. Ehrlich, P. Ueber die specifischen Granulationen des Blutes. *Arch. f. Anat. u. Physiol., Physiol. Abt.*, 1879, 3, 571–579.
2. Wilander, O. Studien über Heparin. *Skandinav. Arch. f. Physiol.*, 1938, 81, suppl. 15, 1–89.
3. Asboe-Hansen, G. The variability in the hyaluronic acid content of the dermal connective tissue under the influence of thyroid hormone. *Acta dermat.-venereol.*, 1950, 30, 221–230.
4. Riley, J. F., and West, G. B. Histamine in tissue mast cells. *J. Physiol.*, 1952, 117, 72P–73P.
5. Harris, H. F. Histology and microchemic reactions of some cells to anilin dyes. *Philadelphia M. J.*, 1900, 5, 757–764.
6. Michels, N. A. The Mast Cells. In: Downey, H. Handbook of Hematology. Paul B. Hoeber, Inc., New York, 1938, 1, 231–372.
7. Paff, G. H., and Bloom, F. Vacuolation and the release of heparin in mast cells cultivated *in vitro*. *Anat. Rec.*, 1949, 104, 45–59.
8. Camble, P. Siderosis and mast cell vacuolation in albino rat after toluidine blue O administration. *Federation Proc.*, 1952, 11, 409–410.
9. Drennan, J. M. The mast cells in urticaria pigmentosa. *J. Path. & Bact.*, 1951, 63, 513–520.

10. Cavallero, C., and Braccini, C. Effect of cortisone on the mast cells of the rat. *Proc. Soc. Exper. Biol. & Med.*, 1951, **78**, 141–143.

11. Asboe-Hansen, G. The mast cell. Cortisone action on connective tissue. *Proc. Soc. Exper. Biol. & Med.*, 1952, **80**, 677–679.

12. Bloom, F. Spontaneous solitary and multiple mast cell tumors ("mastocytoma") in dogs. *Arch. Path.*, 1942, **33**, 661–676.

13. Stuart, E. G. Connective tissue mast-cell responses to bacterial pyrogens, ovalbumin and cortisone. *Anat. Rec.*, 1951, **109**, 351.

14. Baker, B. L., Ingle, D. J., and Li, C. H. The histology of the lymphoid organs of rats treated with adrenocorticotropin. *Am. J. Anat.*, 1951, **88**, 313–349.

15. Smith, D. E., and Lewis, Y. S. Effects of total body x-irradiation on the tissue mast cell. *Proc. Soc. Exper. Biol. & Med.*, 1953, **82**, 208–212.

16. Baeckeland, E. Evolution des mastocytes dans la peau du cobaye à la suite d'implantations sanguines et fibrineuses. *Compt. rend. Soc. de biol.*, 1950, **144**, 1007–1009.

17. Larsson, L. G., and Sylvén, B. The mast cell reaction of mouse skin to some organic chemicals. II. The effect of common organic solvents. *Cancer Research*, 1947, **7**, 680–685.

18. Cramer, W., and Simpson, W. L. Mast cells in experimental skin carcinogenesis. *Cancer Research*, 1944, **4**, 601–616.

19. Compton, A. S. A cytochemical and cytological study of the connective tissue mast cell. *Am. J. Anat.*, 1952, **91**, 301–329.

20. Devitt, J. E., Pirozynski, W. J., and Samuels, P. B. Mast cell resistance to hormonal influence. *Proc. Soc. Exper. Biol. & Med.*, 1953, **83**, 335–337.

21. Schoch, E. P., Jr., and Glick, D. The effect of cold stress, ACTH, cortisone, pyrogen, and nitrogen mustard on tissue mast cells in the skin and subcutaneous tissues of the rat. *J. Invest. Dermat.*, 1953, **20**, 119–132.

[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Tissue mast cells in the mesentery of a control rat. This specimen has been grossly over-stained, causing the cells to appear as solid dark blue-black masses, with no trace of metachromasia. × 700.

FIG. 2. Tissue mast cells in the mesentery of a control rat. These slightly under-stained cells have the characteristics of "normal" mast cells. × 700.

FIG. 3. Tissue mast cells in the mesentery of a control rat. This preparation was washed out excessively in alcohol. The mast cells seem to be poorly filled. Little evidence of "degeneration" can be seen. × 450.

FIG. 4. Tissue mast cells in the mesentery of a control rat. These somewhat over-stained cells show granule conglomeration, vacuolation, and loss of metachromasia. A peripheral ring of clumped granules may be observed in the cell at the right. × 450.

FIG. 5. Tissue mast cells in the mesentery of a control rat. These five over-stained cells have all burst. There are irregular clumps and bars in each cell. × 450.

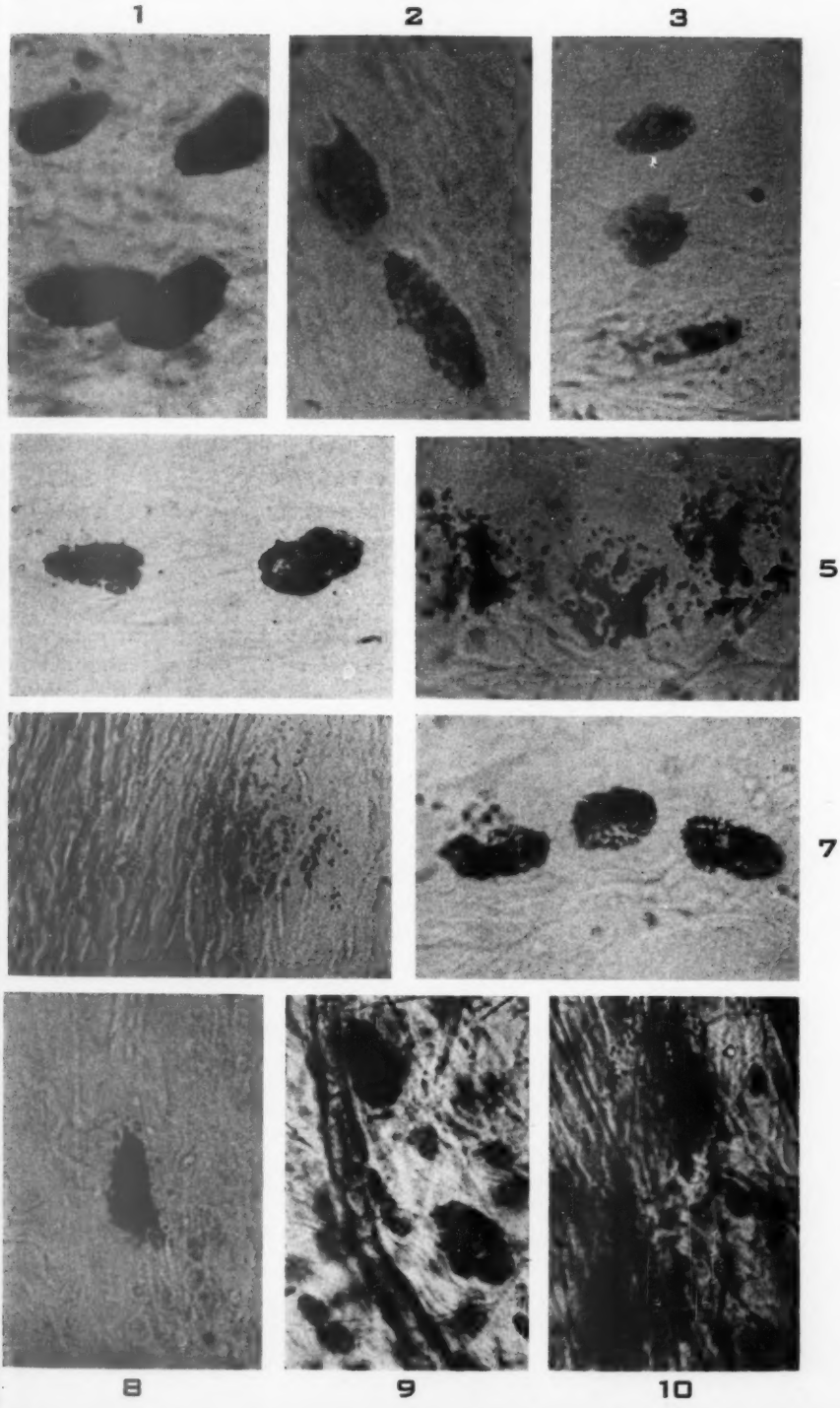
FIG. 6. Tissue mast cells in the mesentery of a control rat. These three cells have burst. In an under-stained specimen such as this, few clumps or agglutinated masses of granules are seen, even in the burst cell. × 450.

FIG. 7. Tissue mast cell in the mesentery of a cortisone-treated rat, showing the irregular granular distribution that is present in all moderately over-stained specimens regardless of treatment. × 450.

FIG. 8. Tissue mast cell in the mesentery of a heparin-treated rat, showing the granule clumping and fraying of the cell outline with scattering of a few granules that can be seen in any moderately over-stained specimen. × 450.

FIG. 9. Tissue mast cells, *in vivo* preparation. These mast cells show the normal appearance always obtained in carefully prepared specimens. × 500.

FIG. 10. Tissue mast cells, *in vivo* preparation. Methanol was dropped on the mesentery just before staining. The mast cells have burst, scattering their granules. × 500.


1
2
3
4
5
6
7
8
9
10